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THE ROLE OF MONOCYTES IN CHRONIC INFLAMMATORY DISEASES

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Front cover: The image shows a monocyte-derived cell from a digested oral mucosa model previously stimulated with LPS and IFN γ . The cells were stained for CD68 (red), MMP12 (green), and DAPI (blue), on a cytospin, original magnification x600. Acquired by Sofia Björfot Holmström.

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The role of monocytes in chronic inflammatory diseases

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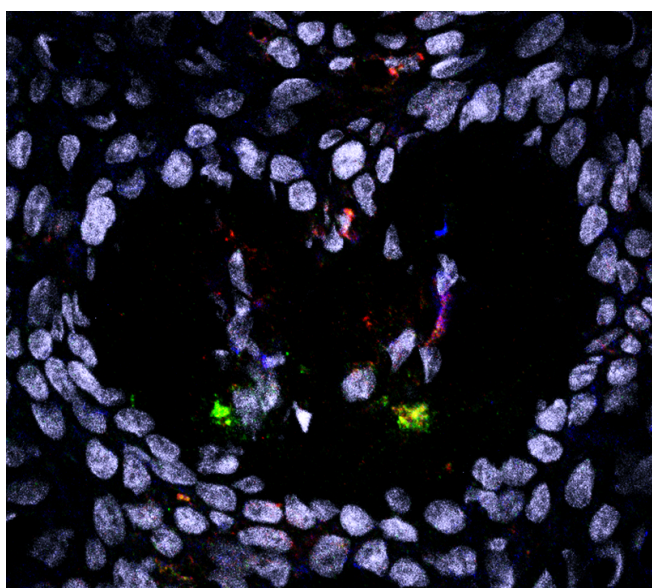
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Acquired by Sofia Björfot Holmström.

To my beloved family

ABSTRACT

Monocytes and monocyte-derived cells are important players in the orchestration of inflammatory reactions in blood and peripheral tissues. However, little is known about the monocyte fate upon entry into human tissues, and current concepts are mainly based on animal models, with the addition of observational studies in humans that often do not allow determining causality. To provide additional understanding of the monocytes and the monocyte-derived cells in tissues, we developed three-dimensional (3D) co-culture models of epithelial tissues with monocytic cells implanted. These 3D tissue models in combination with clinical samples, including blood, saliva and tissue have been the platform for my thesis work on monocytes and monocytes-derived cells in chronic inflammatory diseases.

In **paper I**, we identified increased mRNA expression of *MMP12*, *COX2*, *TNF* and *DC-SIGN*, genes associated with inflammation and tissue remodeling, in gingival tissue from individuals with Periodontitis (PD). The increased production of MMP12 was confirmed at a protein level, and flow cytometry analysis identified CD68⁺CD64⁺CD14⁺ monocyte-derived cells as responsible for increased MMP12 production in tissue. In addition, monocyte-derived cells from PD gingival tissue had a relatively low surface expression of the co-inhibitory molecule CD200R. Similarly, using a multicellular 3D model of oral mucosa with induced inflammation showed increased MMP12 production and reduced CD200R surface expression by monocyte-derived cells. We identified CSF2 as a potent inducer of MMP12, and that treatment of CSF2-stimulated monocyte-derived cells with a CD200 ligand reduced MMP12 production. Thus, this study identified CD200/CD200R as a potential pathway to modulate aberrant inflammatory reactions in order to reduce the subsequent immunopathology and induce resolution of chronic inflammation.

In a follow up study (**paper II**), a larger patient cohort (n=436) was investigated to assess the potential of MMP12, as well as the S100 proteins S100A8/A9 (calprotectin) and S100A12 as salivary biomarkers of PD. We found that MMP12 levels reflect destruction of periodontal structures, while the levels of the S100s reflect periodontal inflammation, and that smoking and age are important to take into consideration in future studies. The presence of other chronic inflammatory diseases did not influence MMP12 and S100 protein levels, however the presence of tumor was associated with an increase in the levels of MMP12 and S100A12.

Paper III was a methodological study, where we further developed the 3D lung tissue model to establish protocols for live imaging analysis of monocytes-derived cell migratory behavior in inflamed tissue. Inflammation was induced by TLR ligand stimulation at the apical side of the lung tissue models, and the level of inflammation was evaluated by flow cytometry, gene expression analysis as well as cytokine secretion. An immunofluorescence live-imagine technique was established to study the migration of the monocyte-derived dendritic cells (DC) in 4D (time, x, y, z) in inflamed lung tissue models.

In **paper IV**, we focused on IL-17A which is a cytokine associated with human chronic inflammatory diseases, and that has been linked to both PD and Langerhans cell histiocytosis (LCH). In LCH, DC-like cells have been described to produce IL-17A, and therefore, we investigated whether blood monocytes had IL-17A-producing capacity. These analyses led to the identification of IL-17A-producing monocytes in patients with LCH, particularly evident in patients with the highest disease activity. In contrast, IL-17A-producing monocytes could not be identified in patients with PD or healthy individuals.

In summary, these studies have contributed to the establishment of new tools to study human monocytes in a tissue milieu, and identified new disease-associated mechanisms and pathways that can be further explored to develop new immunomodulatory treatments for chronic inflammatory diseases.

LIST OF SCIENTIFIC PAPERS

- I. **Sofia Björnfot Holmström**, Reuben Clark, Stephanie Zwicker, Daniela Bureik, Egle Kvedaraite, Eric Bernasconi, Anh Thu Nguyen Hoang, Gunnar Johannsen, Benjamin J. Marsland, Elisabeth A. Boström, Mattias Svensson. Gingival tissue inflammation promotes matrix metalloproteinase-12 production by CD200R^{low} monocyte-derived cells in periodontitis. *The Journal of Immunology* (2017) **199**, doi:10.4049/jimmunol.1700672.
- II. **Sofia Björnfot Holmström***, Ronaldo Lira Junior*, Stephanie Zwicker, Mirjam Majster, Anders Gustafsson, Sigvard Åkerman, Björn Klinge, Mattias Svensson, Elisabeth A. Boström. MMP-12 and S100s in saliva reflect different aspects of periodontal inflammation, *Manuscript*. *contributed equally
- III. Anh Thu Nguyen Hoang*, Puran Chen*, **Sofia Björnfot**, Kari Högstrand, John G. Lock, Alf Grandien, Mark Coles, Mattias Svensson. Live-imaging analysis of human dendritic cell migrating behavior under the influence of immune-stimulating reagents in an organotypic model of lung. *Journal of Leukocyte Biology* (2014) **96**, 481-489. *contributed equally. doi:10.1189/jlb.3TA0513-303R
- IV. Magdalina Luorda, Selma Olsson-Åkefeldt, Desirée Gavhed, **Sofia Björnfot**, Niels Clausen, Ulf Hjalmar, Magnus Sabel, Abdellatif Tazi, Maurizio Arió, Christine Delprat, Jan-Inge Henter, Mattias Svensson. Detection of IL17-A-producing peripheral blood monocytes in Langerhans cell histiocytosis patients. *Clinical Immunology* (2014) **153**, 112-122. doi: 10.1016/j.clim.2014.04.004

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LIST OF ABBREVIATIONS

3D	Three-dimensional
AP-1	Activator protein 1
APC	Antigen presenting cell
BOP	Bleeding on probing
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD200	OX-2 membrane glycoprotein
cDC	Conventional dendritic cell
CLEC	C-type lectin domain
CLR	C-type lectin receptor
COX	Cyclooxygenase
CSF	Colony stimulating factor
CVD	Cardiovascular disease
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C chemokine receptor
DAMPs	Danger associated molecular patterns
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DLL1	Notch ligand delta-like 1
DMEM	Dulbecco's modified Eagle medium
DMFT	Decayed, missing and filled teeth
Dok	Downstream of tyrosine kinase
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FcR	Low affinity Fc receptor
Flt	Fms like tyrosine kinase
FN	Fibronectin
GAG	Glucoseaminoglycan
GCF	Gingival cervical fluid

H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
IDO	Indoleamine 2,3-dioxygenase
IEL	Intraepithelial lymphocyte
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
IL-17RA	IL-17A receptor
IL1Ra	IL1 receptor antagonist
ILC	Innate lymphoid cell
iMac	iPSC-derived primitive macrophage
iNOS	Cytokine-inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
JE	Junctional epithelium
LCH	Langerhans cell histiosytosis
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
Mac-1	Macrophage antigen 1
MAIT	Mucosa-associated invariant T cell
MAPK	Mitogen-activated protein kinase
MCL	Manifest caries lesions
MerTK	Tyrosine-protein kinase MER
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MR1	MHC class I-related protein
MRC	Mannose receptor C type 1
MRP	Myeloid-related protein
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptor
OKF6/TERT-2	TERT-immortalized normal human oral keratinocyte line
PAMPs	Pathogen associated molecular patterns

PBMC	Peripheral blood mononuclear cell
PD	Periodontitis
pDC	Plasmacytoid DC
PDGFD	Platelet-derived growth factor D
PG	Prostaglandin
PI	Plaque index
PI3K	Phosphatidylinositol 3-kinase
PPD	Periodontal probing depth
PRR	Pattern recognition receptor
PTGS2	Prostaglandin-endoperoxide synthase 2
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation endproducts
RANK	Receptor activator of nuclear factor κ B
RasGAP	Ras GTPase activating protein
RT-qPCR	Real time quantitative reverse transcription polymerase chain reaction
SE	Sulcular epithelium
SIRP α	Signal regulatory protein alpha
T _C	Cytotoxic T cell
TCR	T cell receptor
T _{FH}	Follicular helper T cell
TGF	Transforming growth factor
T _H	Helper T cell
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAP	Tartrate-resistant acid phosphatase
T _{reg}	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
T _{RM}	Tissue resident memory T cell

VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WB	Western blot

1 INTRODUCTION

1.1 AN OVERVIEW OF THE IMMUNE SYSTEM

The host is protected through different lines of defense, starting with physical barriers, such as skin and mucosal tissues. All tissue barriers are equipped with specialized mechanical properties, anti-microbial compounds, and inflammatory mediators to protect against invading pathogens. The commensal microbiota is layered on top of these surface barriers to compete with pathogens for nutrients and space, and is separated from the epithelium by a thin layer of mucus, which also acts as a barrier for pathogens. The epithelium is covering all surfaces of the body, and a basement membrane separates the epithelium from the underlying connective tissue, which is vascularized and composed of a wide variety of cells including the white blood cells (leukocytes, immune cells). The immune cells belong either to the innate or the adaptive immune system, and both are present within tissues where they communicate with each other as well as with the tissue constituent cells. Innate immune cells of both lymphoid and myeloid origin are located within epithelial tissues to rapidly combat infections, as well as maintain tissue homeostasis. This thesis focuses on myeloid mononuclear phagocytic cells, which function as an arm between the innate and adaptive immunity, as well as conductors of immune reactions within the innate immune systems. These cells are equipped with receptors called pattern recognition receptors, (PRR), that recognize different pathogen and danger associated molecular patterns (PAMPs and DAMPs). Upon ligation, these receptors trigger cellular activation leading to engulfment of extracellular material, including infectious agents, which are processed into peptides for presentation to T lymphocytes. In addition, activated phagocytic cells respond immediately to infectious assaults or injury with production of inflammatory mediators. Besides combatting infections, the inflammatory cells and their secreted products can cause tissue damage, and when there is a lack of control, this can lead to irreversible tissue destruction.

1.1.1 The innate immune system

The innate immune system is able to respond rapidly to infections, and include epithelial cells and stromal cells, such as fibroblasts, the complement system, as well as lymphoid and myeloid immune cells (1). Epithelial cells form a barrier with tight intercellular junctions and in addition they can sense infections with PRR to rapidly respond with the production of antimicrobial peptides as well as cytokines to activate tissue-resident immune cells (2, 3). Fibroblasts are the most prevalent cells within lamina propria, with functions ranging from extracellular matrix (ECM) production and remodeling, production of growth factors and inflammatory mediators to alert and recruit immune cells (4). Residing within epithelial barriers, myeloid cells scavenge and clear pathogens from the lumen of certain organs, such as the intestine and lung (5). The process by which the pathogens are engulfed is named phagocytosis, a process discovered by Elie Metchnikoff who was awarded the Nobel prize in 1908 for his discoveries (6). Once the pathogen has managed to pass the epithelial barrier into the lamina propria, several cell types, including the myeloid immune cells, contribute to

mount a strong inflammatory response. Mast cells, which are also of myeloid origin, are located within tissues and rapidly release inflammatory mediators such as histamine, proteases and leukotrienes, mediating increased vascular permeability and inflammation, upon encounter of multicellular pathogens, but also to allergens (7). The granulocytes are polymorphonuclear myeloid leukocytes comprised of three different subtypes, the neutrophils that are important for bacterial defense, and the eosinophils and basophils that are implicated in parasite defense. Upon infection, the neutrophils are rapidly recruited to the infected tissues where they engulf bacteria and release their granules and extracellular traps to kill pathogens (8).

In addition to the granulocytes, there are mononuclear phagocytic cells including monocytes, monocyte-derived cells, dendritic cells (DCs) and tissue-resident macrophages that are able to respond to different types of infections due to their broad expression of PRR such as toll like receptors (TLRs) and C-type lectin receptors (CLR) (9). Among monocytes, macrophages, and DCs there are different subsets with overlapping and distinct functions. The family of DCs is particularly important for their superior endocytic function and capability to process engulfed proteins into peptides that can be presented on human leukocyte antigen (HLA), also known as major histocompatibility complex (MHC) molecules, to T lymphocytes, which are cells of the adaptive immune system (10). The DCs are divided into conventional DCs (cDCs, sometimes referred to as myeloid DC) that can be further subdivided, and into the plasmacytoid DCs (pDCs) (11). Monocytes and macrophages also share the ability to interact with T cells, mostly for the purpose of potentiating killing capacity of pathogens as well as in the resolution of inflammatory reactions (12, 13). Besides activation by PRR sensing, the immune cells can also sense stress caused by a deviation in regulatory variables such as oxygen levels, cell and ECM composition, and level of nutrients (14). This additional activation mechanism is also thought to be important in the distinction between pathogens and the commensals, as well as to sense multicellular pathogens (15).

As part of the innate immune system are also cells of lymphoid origin. This includes for example natural killer (NK)-cells that are able to recognize virus infected cells or cells that differ from the host cells due to lack of HLA class I expression, known as “missing-self”, and are important in the protection of intracellular infections and tumors (16). In addition to NK cells, there are three groups of invariant lymphocytes that belong to the innate immune system, namely NK T cells, gamma delta ($\gamma\delta$) T cells, and mucosa-associated invariant T cells (MAITs). These cells are able to recognize different bacterial products and metabolites, and can sense antigens presented by infected cells through different cluster of differentiation (CD) 1 molecules and the MHC class I-related (MR1) protein, rather than via HLA molecules (17). Upon stimulation, these cells produce cytokines and chemokines important to activate and recruit innate and adaptive immune cells to the site of infection and inflammation. More recently another group of innate cells, namely the innate lymphoid cells (ILCs) were discovered and now comprise three distinct populations that populate lymphoid organs and mucosal tissues (18). Among other functions the ILCs promote barrier defense functions and

tissue homeostasis (18, 19). In addition to the innate immune cells, there is the complement system that promotes activation of immune cells, removal of antigen-antibody complexes and dead cells, opsonization of bacteria to facilitate phagocytosis, and pore formation in the membrane of pathogens (20).

To summarize, the role of the innate immune system is to form a barrier to protect us from invading pathogens. When the mechanical barrier is damaged, the innate immune system responds within minutes to hours with a broad range of weapons to clear pathogens and damaged cells, and to recruit and activate other immune cells. This response can if it is uncontrolled for too long lead to irreversible tissue destruction, which can be the case in chronic inflammatory diseases. In addition, innate immune cells are constantly communicating with the adaptive immune cells, where innate immune cells sense pathogens or tissue damage, and interact with adaptive immune cells to mount antigen-specific immune responses (15, 21). The adaptive immune cells in turn produce a second level of cytokines and in the case of B cells antigen-specific antibodies. These cytokines and antibodies induce different effector mechanism in cells such as macrophages, epithelial cells, fibroblasts and granulocytes, leading to potentiated immune responses (15). A well-coordinated immune response can destroy invading pathogens, keep tissues intact and induce processes of wound healing.

1.1.2 The adaptive immune system

In contrast to the innate immune system, the adaptive immune system is an evolutionary event found only in vertebrates (22). T and B cells, which are adaptive immune cells, are able to rearrange their gene segments upon activation after encountering antigens. A part of the adaptive immunity is the T-cell mediated cellular response, which requires antigen presentation by other cells on HLA class I or II molecules. T cells are divided into T helper (T_H) cells, expressing CD4, and the cytotoxic T (T_C) cells expressing CD8, in addition to CD3 that is expressed by all T cells. Activation of the T cells and rearrangement of their genes encoding the T cell receptor (TCR) occur in the lymphoid organs upon antigen presentation by professional antigen-presenting cells (APC). Antigen-specific activation together with additional signals (co-stimulation and cytokines) provided by the APC, induce T cell activation and clonal expansion, resulting in T cells with specific functions (23). Activated $CD8^+$ T cells gain killing capacities and are referred to as cytotoxic T cells (T_C) that target infected or transformed cells expressing antigens recognized as foreign (24). $CD4^+$ T_H cells on the other hand are activated and differentiate into different types of effector T_H cells, specifically T_H1 , T_H2 , T_H17 , and the recently described T_H9 and T_H22 cells, as well as regulatory T cells (T_{reg}) and follicular helper T cells (T_{fh}), all with distinct functions to control the immune responses and maintain tissue homeostasis, either by suppression or activation of inflammatory pathways (25-27). The different T cells can also become memory cells, including the tissue resident memory T cells (T_{RM}) that reside in barrier tissues to rapidly mount a specific immune response to a certain pathogen (28).

The adaptive immunity also includes a humoral response, consisting of antibody-producing B cells. The activation of B cells takes place in secondary lymphoid organs where the naïve B cells encounter antigen, independent or dependent on CD4⁺ T_H cell interactions (TCR/HLA-II, CD40/CD40L and cytokines) (29). Activation of the naïve B cells will lead to either memory B-cells that are able to present antigens or into plasma cells that produce different types of antibodies. In secondary lymphoid tissues the activated B cells can form germinal centers, a process important for antibody affinity maturation, the generation of memory B cells as well as formation of long-lived plasma cells (29). Antibodies have broad effects on several parts of the immune system, e.g., by activating immune cells to release effector molecules, tag pathogens by a mechanism called opsonization which facilitates phagocytosis, neutralize toxins and microbes, and activate the complement system (30). Taken together, the adaptive immune response requires education and activation by specific antigens and cytokines to mount a powerful and specific response aimed to target certain pathogens. If the education fails and the adaptive immune cells recognize a self-antigen or innocuous environmental antigens, autoimmune or allergic diseases, respectively, can be the consequence.

1.2 CHRONIC TISSUE INFLAMMATION

Chronic inflammatory diseases occur worldwide, and include heterogeneous types of diseases in various organs. The inflammation can either be sterile or from infectious triggers, associated with either autoimmune diseases or chronic infectious and inflammatory diseases. Autoimmune diseases are linked to certain self-antigens that trigger an adaptive immune response, while chronic inflammatory diseases without certain antigens linked to the pathogenesis are more complicated. Such chronic inflammatory diseases are often multifactorial, with other systemic diseases, environmental factors (smoking, diet, stress, allergens, medication), and genetic susceptibility linked to the on-set of the diseases, and involve various cell types. In addition, an altered commensal microbiota is speculated to be an important trigger in several chronic inflammatory diseases such as Periodontitis (PD) and Crohn's disease (31, 32). However, the inflamed tissues facilitate the colonization of alternate microbes, such as anaerobic bacteria in the case of PD, and therefore this may result from the disease rather than being the cause (33). Chronic inflammation can also result from T cell responses to innocuous antigens, resulting in allergic diseases (28). Depending on the type of chronic inflammation, different lymphoid and myeloid cell subsets are implicated in the inflammatory reactions and disease progression. Characterizing the tissue environment as well as the cell subsets associated to the progression of the chronic inflammatory diseases, can contribute to identify new potential targets for immunomodulation.

1.3 MONOCYTES AND MONOCYTE-DERIVED CELLS

This thesis focuses on blood monocytes and their tissue equivalents that we refer to as monocyte-derived cells, and how these cells are implicated in the pathogenesis of chronic inflammatory diseases. I will start with introducing the blood monocytes and their classification and function in blood as well as the processes by which they are recruited into tissues. When entering the tissue, monocytes can remain as effector cells or undergo differentiation to become a heterogeneous population of monocyte-derived cells. The differentiation of monocytes in tissue varies depending on the tissue microenvironment, which I will describe in more detail below. I will end with a brief introduction on the tissue-resident macrophages that originates from an embryonic precursor, but overlap functionally and phenotypically with the monocyte-derived cells in tissues. Over all, monocytes are important for the balance between immunity and tolerance in order to maintain tissue homeostasis, and they are able to initiate, maintain and resolve inflammatory reactions. Considering their diverse roles in the different stages of the inflammatory reaction, they serve as potential treatment targets, by modifying disease-associated functions.

1.3.1 Monocytes

1.3.1.1 Origin and classification

Monocytes continuously derive from hematopoietic stem cells in the adult bone marrow through several developmental steps called hematopoiesis (Figure 1), after which they enter the peripheral blood where they constitute approximately 10-15 % of the leukocytes (34-36). During the last decades the understanding of the monocytes have increased tremendously, from the theory of monocytes as a homogenous precursor for tissue macrophages (37), to the knowledge that they represent a heterogeneous population of cells with a broad range of distinct functions (11, 38, 39). Proposing that the prefix mono not properly reflect this heterogeneous and highly plastic cell type. Advanced flow cytometry techniques allowing analysis of up to 32 different markers in one sample, as well as the rapidly developing single cell sequencing techniques in combination with advanced humanized animal models (36, 40), have contributed to the discrimination of the different monocyte subsets and their functions as well as their developmental relationships.

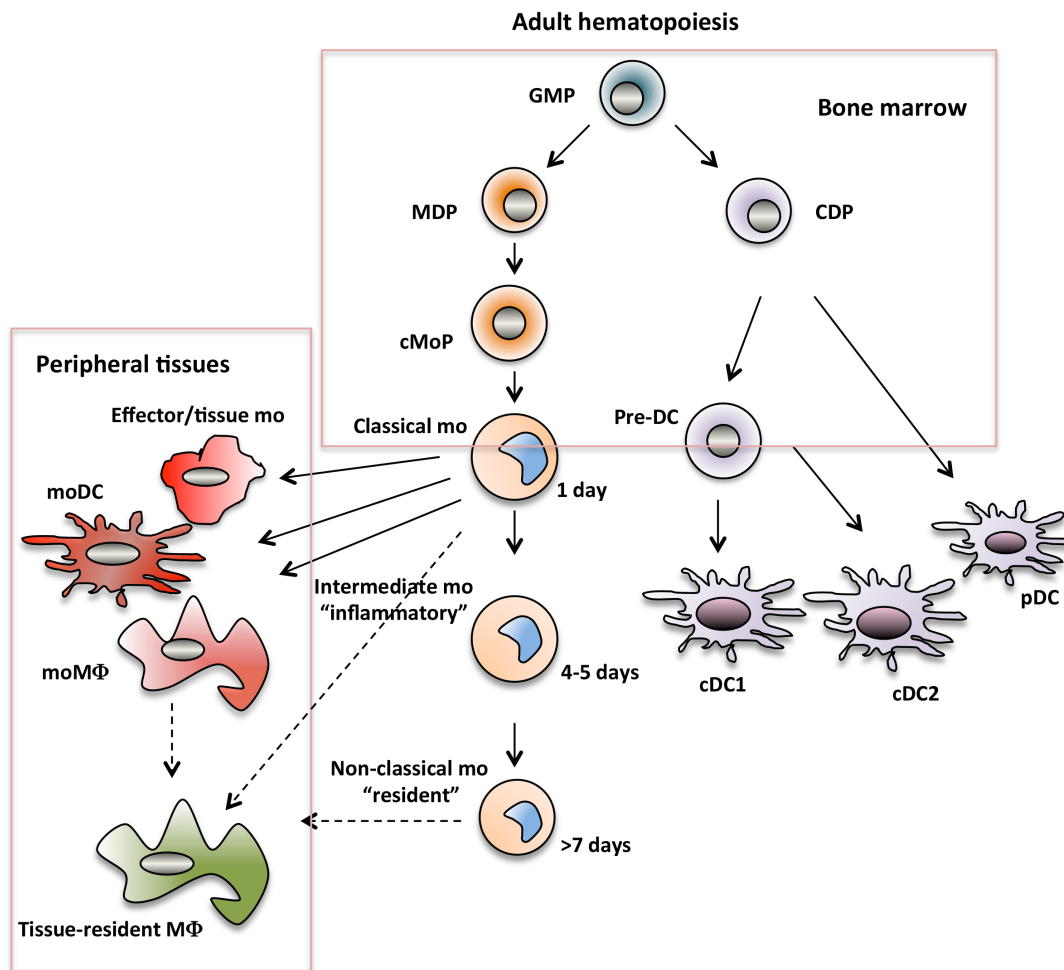


Figure 1. Schematic illustration of adult hematopoiesis. In bone marrow the granulocyte/macrophage progenitor (GMP) give rise to two distinct progenitors, the macrophage/dendritic cell (DC) progenitor (MDP) and the common DC progenitor (CDP). The CDP further give rise to the pre-DC that is the precursor for the conventional DC (cDC). In addition the CDP give rise to plasmacytoid DC (pDC). The MDP further differentiate into the common monocyte progenitor (cMoP), which is the precursor for blood monocytes. The cMoP first differentiates into classical monocytes, which are the precursor for intermediate monocytes that in turn differentiates into the long-lived non-classical monocytes. The classical monocytes are recruited to tissues where they differentiate into effector cells and monocyte-derived dendritic cells (moDC) and macrophages (moMΦ). It is speculated that the classical and non-classical monocytes, as well as the moMΦ can replenish the embryonically derived tissue-resident MΦ. *Illustration by Sofia Björnfor Holmström.*

Today three distinct subpopulations are described based on their expression of the lipopolysaccharide (LPS) co-receptor CD14 (41) as well as the low affinity Fc receptor (FcR)III, CD16 (42) (Figure 2), namely the classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, and non-classical $CD14^{-}CD16^{+}$ monocytes (11, 39, 43). Recent mass cytometry (CyTOF) analysis suggests expanding the panel of markers including also C-C chemokine receptor (CCR) 2, CD11c, HLA-DR and CD36 to more precisely discriminate the different monocyte subsets (44). Depending on the subset specificity, monocytes express a broad array of various receptors for sensing of pathogens, apoptotic cells, and inflammatory mediators, as well as for the adhesion to endothelial cells (43, 45, 46).

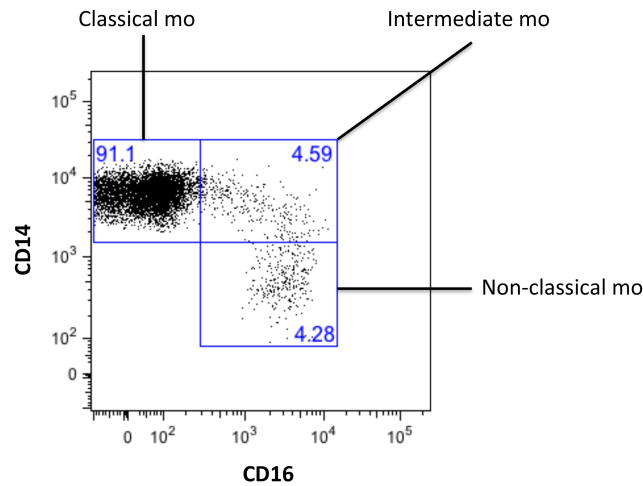


Figure 2. Flow cytometry gating on the three human monocyte subsets based on their differential surface expression of CD14 and CD16. Doublets, dead cells, HLA-DR-negative cells as well as B-cells (CD14-negative), were excluded prior to this gating.

1.3.1.2 Classical monocytes

The $CD14^{++}CD16^{-}$, classical monocyte subset comprises 85-95% of the monocytes in peripheral blood in health. Recent findings suggest that this subset is the only monocyte population found in the bone marrow, and is recruited to peripheral blood in a CCR2 dependent manner (36, 47). The $CD14^{++}CD16^{+}$ monocytes only stay in circulation for one day until they undergo differentiation or are cleared from the circulation either by extravasation into tissues or by death (36, 48). The classical monocytes express a broad array of inflammatory chemokine receptors, e.g. CCR1, CCR2 (Figure 3), CCR5, CCR7, C-X-C chemokine receptor (CXCR) 1, and CXCR2, making them highly responsive to signals that recruit them to sites of infection or tissue injury (49). They express different PRRs, such as TLRs, CLRs and nucleotide-binding oligomerization domain-like receptors (NLRs) to sense extracellular and intracellular pathogens (45). In response to PRR ligation, they produce chemokines and proinflammatory cytokines, such as C-C motif chemokine ligand (CCL) 2, CCL3, CCL5, interleukin (IL)-8/CXCL8, IL-10 and IL-6 (43, 49, 50).

Compared to the other monocyte subsets, the classical monocytes express higher levels of CLEC4D, CD33, CD99, CD163, CD1d, the adhesion molecules L-selectin (CD62L) (Figure 3) and CD11b/Mac-1, the Fc receptors CD32 and CD64, while they display lower HLA-DR, CD11c, and colony stimulating factor (CSF) 1 receptor (CSF1R/CD115) expression compared to the other subsets (36, 45, 49). Gene expression analysis revealed that 942 and 1456 genes were differently expressed by the classical monocytes compared to the intermediate and non-classical monocytes, respectively, while only 256 genes differed between the latter two, which is in line with the results by Zawada et al. (39, 49). Among the highest expressed genes in the classical monocytes are the antimicrobial S100 proteins, S100A8 (calgranulin A or myeloid-related protein 8 (MRP-8), S100A9 (calgranulin B or MRP-14), and S100A12 (calgranulin C) (39, 49).

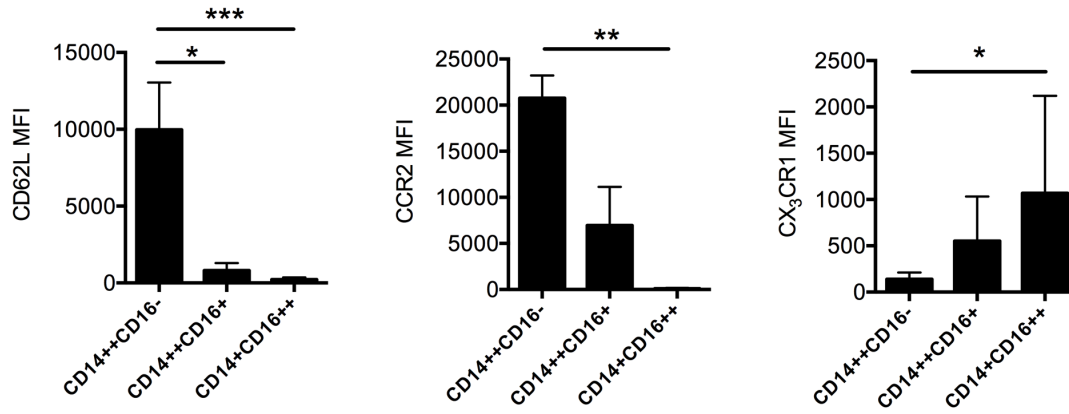


Figure 3. CD62L, CCR2 and CX₃CR1 median fluorescence intensity (MFI) expression on the three monocyte subsets from five donors. Data are presented as mean \pm SD, and the Friedman test with Dunn's multiple comparison test was applied to analyzed statistical differences, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Based on studies in mouse models, the classical monocytes are suggested to migrate into tissues under steady state conditions, as well as the subsequent migration into lymph nodes where they can present antigens (13, 51, 52). Besides antigen presentation the classical monocytes may also activate CD8⁺ T_C cells and NK cells via production of IL-18 and IL-15 (53). Though, the main function of classical monocytes is suggested to be as effector cells and contribution to the monocyte-derived cell pool in inflamed/infected tissues. In line with this, classical monocytes are shown to be the most efficient monocyte subset at phagocytosis and together with their high expression of antimicrobial peptides, this subset have an important role in the innate defence against microbial pathogens (39, 43).

1.3.1.3 Intermediate monocytes

The origin of the intermediate monocytes has been under extensive investigation, to determine if it is a separate developmental line or if these cells originate from other monocyte subsets. For several years, theories on the classical monocytes as precursors for the other monocyte subsets have been proposed. This concept has, however, been difficult to prove, but recent animal models have shown that this may indeed be the case (48). In addition, newly developed techniques have enabled this to be confirmed in a human *in vivo* study where the monocytes were traced and depleted (36, 54). By monitoring the repopulation in circulation, it was found that a proportion of the classical monocytes, which were the once arriving first, are precursors of the intermediate monocytes, which in turn matures into the more long-lived non-classical monocytes (36, 48). Also, a mouse model showed differentiation of the classical monocytes into the resident monocyte subset (Ly6C^{low}). This was found to be Notch2 dependent, via notch ligand delta-like 1 (DLL1) signalling by endothelial cells (55). DLL1 signalling together with the transcription factor Nr4a1 have also been reported to be important for the survival of the resident monocyte subset (56, 57). In mice though, the monocytes are only composed of two distinct subsets, the Ly6C^{high} resembling classical monocytes, and the Ly6C^{low} resembling the CD16-positive monocytes (46), and therefore it is not possible to draw definite conclusions that this is the case for both

the human intermediate and non-classical monocytes. The CSF1R signalling is also important for the generation and survival of the intermediate as well as the non-classical monocytes (58). In line with this, antibodies against CSF1 diminish intermediate and non-classical monocytes in peripheral blood in patients with rheumatoid arthritis (RA) (59). The intermediate monocyte subset shares phenotype and functional characteristics with both the classical and non-classical monocytes, though more closely related to the non-classical subset (49). As the intermediate monocyte subset expresses high levels of HLA-DR, CLEC10a and CD40, these monocytes are often referred to as “inflammatory” monocytes (49). This is further strengthened due to their increase in numbers and association to several chronic inflammatory diseases (50, 60-66).

These monocytes express intermediate levels of the proinflammatory chemokine receptors, except for CCR5, which they have high expression of, and in addition they up-regulate CX₃CR1 (Figure 3), potentially mediated via CCL2 ligation (65, 67). The CD14⁺⁺CD16⁺ monocytes are thought to stay in circulation where they are able to rapidly respond to signals of damage or infection by the initiation of inflammation via the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF), IL-6 and IL-1 β (43, 45, 50, 68). In addition, they are able to phagocytose micro particles (45). It is suggested that this monocyte subset can migrate to lymph nodes to present antigens, supported by their high HLA-DR and CD74 expression (39, 69). Another function associated to this subset of monocytes is their potential involvement in angiogenesis, identified by their gene and protein expression of the angiopoietin receptor Tie-2, the vascular endothelial growth factor (VEGF) receptor 2, and endoglin (39, 70-72).

1.3.1.4 *Non-classical monocytes*

The non-classical monocytes are the most long-lived subsets, able to stay in circulation for around a week or longer until they undergo cell death (36). This subset acts as a guardian of the blood circulation by patrolling the vessel walls in a lymphocyte function-associated antigen (LFA)-1 dependent manner to sense damage and infections (Figure 4) (43, 56, 73, 74). Their crawling and patrolling behaviour might mask the actual number and phenotype of this subset of monocytes in peripheral blood draws. Besides low CD14 expression and high CD16 expression, this subset also expresses high levels of CX₃CR1 (figure 3), CD115, CD294, siglec10, CD43, SIRP α , CD11a, and CD11c (39, 45, 49). Distinct functions by this subset include the production of IL1 receptor antagonist (IL1Ra) in response to bacterial stimuli, while the sensing of nucleic acids and viral infection by intracellular TLR3, TLR7 and TLR8 induce production of proinflammatory cytokines and type I interferons (IFN) (43, 45). In addition, they are also able to produce cytokines such as IL-6, IL-8, and IL-10 in response to stimuli (43, 49). Their SIRP α expression is shared with tissue-resident macrophages and together with the ability of this subset to differentiate into monocyte-derived macrophages under the stimuli from growth factors *in vitro*, this subsets might be a blood counter-part to the “wound-healing” tissue-resident macrophages that are involved in

the resolution of inflammation and at steady state conditions (45, 73). It is also speculated that the non-classical monocytes extravasates into tissue at the resolution phase of inflammation to support the tissue-resident macrophages in restoring damaged tissues (Figure 4) (75).

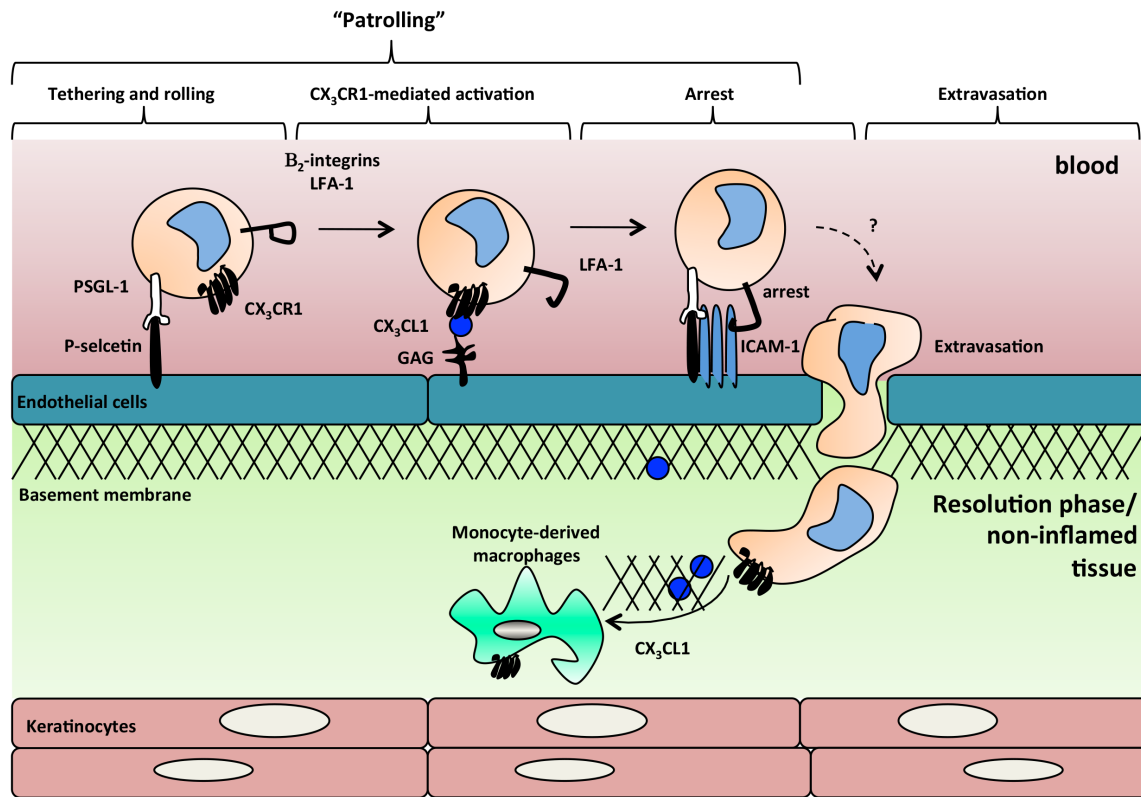


Figure 4. Schematic illustration of the patrolling of the vessel walls in a LFA-1 dependent manner by the non-classical monocyte, as well as the mechanism by which they may be recruited to the non-inflamed tissue or in the resolution phase where they are assumed to assist the tissue-resident macrophages in the wound healing process. The recruitment and survival of the monocytes in this scenario is speculated to be CX₃CR1/CX₃CL1 mediated. PSGL-1, P-selectin glycoprotein ligand-1; ICAM-1, Intercellular adhesion molecule 1; GAG, glycosaminoglycan; LFA-1, lymphocyte function-associated antigen 1; CX₃CR1, C-X₃-C motif chemokine receptor 1; CX₃CL1/fractalkine, C-X₃-C motif chemokine ligand 1. *Illustrated by Mattias Svensson and Sofia Björnfor Holmström.*

1.3.1.5 Monocyte recruitment to tissues

Recruitment of immune cells to the site of infection is directed via chemokines and their corresponding surface receptors, which production and expression is induced by infectious and inflammatory stimuli (76). CCR2, mainly expressed by the classical monocytes and the expression of its ligands on glycosaminoglycans (GAGs) on tissue and endothelial cells, is pivotal in the emigration of monocytes from bone marrow to blood as well as further recruitment into tissues (Figure 5) (47, 52, 77, 78). CCR1 and CCR5 are also suggested to be involved in the recruitment of monocytes to inflamed tissues (78). In addition to chemokine-mediated recruitment, monocyte trafficking into non-lymphoid and lymphoid tissues depend on different adhesion molecules (79). The binding of CD62L to its ligands on endothelial cells is crucial for the extravasation into tissues (Figure 5) (13). Other adhesion molecules

such as the Very Late Antigen-4 (VLA-4) that is activated when stimuli are present, or the $\alpha_M\beta_2$ /Mac-1 including the monocyte/macrophage marker CD11b, are also important for the extravasation of monocytes (Figure 5) (80, 81). Contact with the endothelium induces the up-regulation of HLA-DR on monocytes, facilitating their ability to present antigens (13).

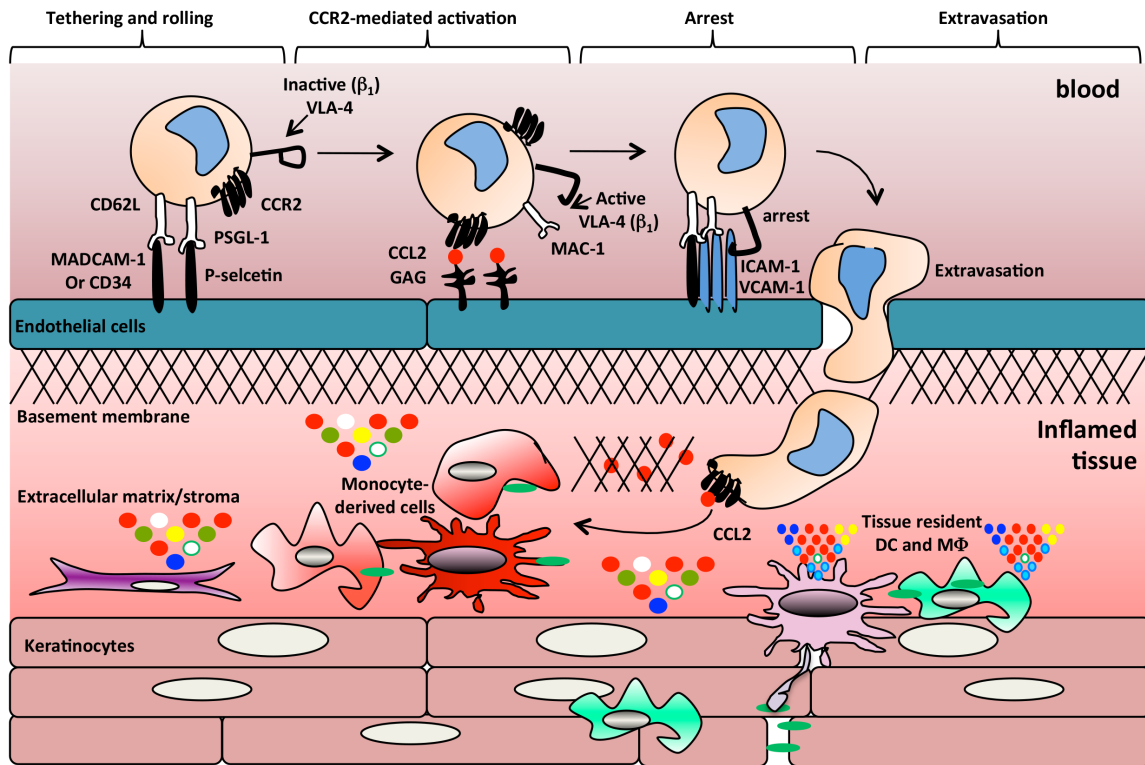


Figure 5. Illustration of the extravasation of monocytes from circulation into inflamed tissue, where they can differentiate into diverse effector cells, depending on the microenvironment. The tethering is mediated via different selectins and their ligands. The binding of inflammatory C-C chemokine receptor (CCR) 2 to its ligand CCL2, presented on glycosaminoglycans (GAGs) by endothelial cells, activates very late antigen (VLA)-4. The activated VLA-4 and the macrophage-associated antigen (MAC)-1 bind to intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which mediates arrest of the monocytes to the endothelium and is followed by the extravasation. CD62L, L-selectin or SELL; MADCAM-1, mucosal vascular addressin cell adhesion molecule 1. *Illustrated by Mattias Svensson and Sofia Björnfor Holmström.*

One of the earliest events of tissue inflammation is the massive infiltration of neutrophils, but also the recruitment of monocytes into tissue is rapid and can occur independently of neutrophils (82). It has even been suggested that monocytes are required before the recruitment of neutrophils, and under certain specific circumstances, this can certainly be the case (83). Under several inflammatory and infectious conditions, or upon challenges to the commensal microbiota due to breaches in epithelial barrier integrity, there is an influx of classical monocytes (84, 85). In addition to chemokines and cytokines, matrix metalloproteinases (MMPs), which are a group of enzymes that degrade ECM components, are important for the monocyte movement within tissues (86). The end products from the cleaved extracellular matrix, called matrikines, can also recruit immune cells to the site of tissue damage (87, 88). There are also several processes to attenuate migration of monocytes in the resolution phase of the inflammation, such as the IL-10 production by monocyte-

derived cells and tissue-resident macrophages, which reduces the recruitment of monocytes via the inhibition of CCL2 production (89). It is challenging to define the subset origin of monocytes entering tissue without previous labelling, since the non-classical monocyte markers CD16 and CX₃CR1 are rapidly up regulated on monocyte-derived cells in tissue as well as on *in vitro* cultured monocytes in the presence of CSF1 (Figure 6) (90). In addition, markers of classical monocytes such as CCR2 can be induced on the intermediate monocyte by infectious stimuli, suggesting that the migratory phenotype of the monocyte subsets differ in steady state and inflammation. The characteristic HLA-DR^{high} expression on the intermediate monocytes can also be induced on the classical monocytes when they adhere to activated endothelial cells (13).

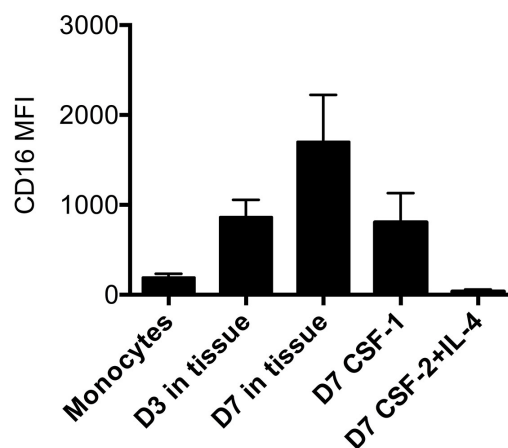


Figure 6. Analysis of CD16 expression on freshly isolated monocytes, as well as their expression of CD16 after differentiation in the oral mucosa model at day 3 (D3) or day 7 (D7) after implantation. In parallel, the monocytes were also cultured in the presence of the growth factor CSF1 for macrophage-like differentiation, or CSF2 and IL-4 for differentiation into monocyte-derived DCs. Data is representative of four donors, and is presented as mean \pm SD.

Taken together, the rapid recruitment of monocytes to the site of infection or injury is crucial for inhibiting the dissemination of infections, and is mediated via the clearance of pathogens, recruitment of other circulating immune cells as well as the activation of the adaptive immune cells. To avoid too much tissue damage, as a result of the initial influx of immune cells and their inflammatory reactions, control of the inflammation is important. When this control is insufficient, allowing the inflammation to continue, immunopathology can arise and as a result chronic inflammatory diseases develop.

1.3.2 Monocyte-derived cells

1.3.2.1 Differentiation

Upon entering tissues the environment dictates the fate of the monocytes. In inflamed tissue, depending on the time after infection as well as the extent of the infectious stimuli, monocytes are suggested to commit to three distinct differentiation pathways (91). At the start of the infection when the microbial load is high, monocytes preferably acquire the role

as effector monocytes resembling activated tissue-resident macrophages (91). This also includes the inflammatory TNF-, and cytokine-inducible nitric oxide synthase (iNOS)-producing monocyte-derived “Tip” DCs (91-93). The effector cells are effective in killing of pathogens and also in producing inflammatory cytokines to instruct other immune cells. In this acute phase, the tissue-resident macrophages can decrease in number, likely via necroptosis or via their migration to lymph nodes, followed by a return in the resolution phase, implicating the importance of the monocytes in this phase of the inflammatory reaction (94, 95). Though when lower concentrations of the stimuli occur, as well as in steady state, monocytes may stay as monocytes or differentiate into monocyte-derived DCs, both able to activate T-cells (13, 45, 91, 96-98). In an *in vitro* study it was showed that only the classical monocytes were able to differentiate into monocyte-derived DCs in the presence of CSF2 and IL-4, and none of the monocyte subsets could acquire a pDC phenotype in the presence of IL-3 and fms like tyrosine kinase (Flt) 3 ligand (45).

If the monocytes arrive to the tissue at a later time-point when the infection is under control, the monocytes can also differentiate into monocyte-derived macrophages that facilitate clearance of debris and apoptotic cells as well as promote tissue repair (75, 91, 99). In the dermis compartment of the skin, and in the lung and gut mucosa, as well as in the spleen and heart, monocytes are shown to partly or completely replenish the embryonically derived tissue-resident macrophages (100-102). As mentioned before, it is suggested based on mouse models that mainly the classical monocytes extravasates into tissues, though it is speculated that the non-classical monocytes migrate into tissue during steady state and in the resolution phase of inflammation where they differentiate into macrophage-like cells. In line with this, several studies on inflammatory diseases observe increased number of CD16⁺CX₃CR1⁺ monocyte infiltration (62, 63, 103). However, it is shown that the CCR2⁺ classical monocytes change their phenotype when they enter the inflamed tissue into CCR2^{lo}CX₃CR1⁺ and facilitate wound healing (84, 90).

To support the theory of monocytes as effector cells in inflammation and infections, other studies also suggest that monocytes can differentiate into monocyte-derived effector cells resembling inflammatory macrophages in inflamed tissues, while they are suggested to differentiate into cells resembling the steady state tissue-resident macrophages with remodelling functions in the resolution phase (52, 84). *In vitro* differentiation of monocytes in the presence of the macrophage growth factor CSF1 or the DC growth factors CSF2 and IL-4 identified CD14, CD32 and CD64 as markers of undifferentiated monocytes and monocyte-derived macrophages, on which they were somewhat increased (104-106). Tyrosine-protein kinase MER (MerTK) was found to be a marker specifically expressed by the monocyte-derived macrophages, and is involved in the efferocytosis via binding to phosphatidylserine on apoptotic cells as well as in homeostasis (104-106). *In vitro* monocyte-derived macrophages and DCs express a wide array of MMPs that are important for their migration and tissue remodelling, though in the presence of stimuli the production is induced and if unregulated the MMPs can cause tissue damage (107, 108). Tissue inhibitors of

metalloproteinases (TIMPs) are natural inhibitors for the MMPs (109). In the presence of CSF1 and receptor activator of nuclear factor κ B (RANK) ligand as well as microbial products and cytokines such as TNF, the classical monocytes can also differentiate into multinucleated osteoclasts, which are macrophage-like cells specialized in bone degradation (110, 111).

1.3.2.2 Polarization

Monocyte-derived cells and tissue-resident macrophages are highly plastic cells, and the process by which they change their phenotype following environmental changes, is referred to as polarization. The macrophages have been classified into classically and alternatively activated (112, 113). Later as well as in parallel, based on *in vitro* stimulations with distinct cytokines the macrophages were described to be either M1 (LPS + IFN γ , or with CSF2) or M2 (IL-4) polarized, a terminology adopted from the T cell field of research with T_H1 and T_H2 immunity (114-116). Later the M2 polarisation was further divided into M2a (IL-4 or IL-10), M2b (IL-13) and M2c (immune complexes + LPS) (117). Markers for the different *in vitro* differentiated phenotypes have been identified, such as CD80, CD64 and CD40 for the M1 and CD163, CD206 (MRC1, mannose receptor C-type 1), and CD200R for the M2 macrophages (118, 119). The CD80 and CD40 are co-stimulatory receptors involved in the activation of T cells. In contrast, the markers on the “M2” cells are linked to scavenging (CD163, CD206) and immune inhibition (CD200R) (120). The CD200/CD200R pathway is important in the control of inflammatory reactions and maintenance of homeostasis, and is a perfect example of the immune cell-tissue cross talk. The expression of OX-2 membrane glycoprotein (CD200) mainly by epithelial cells, mesenchymal stem cells and fibroblasts, provide inhibitory signals to CD200R-expressing immune cells (121-123). In addition to the cytokines IL-13, IL-4, and IL-10, CSF1 also have immunosuppressive functions on the monocyte-derived cells and macrophages (124).

In 2014, guidelines were introduced for *in vitro* polarization studies and efforts have been made to the understanding on how a broad array of different stimulus influences macrophage polarization, suggesting the correlation of certain stimuli with a specific phenotype (104, 125). Next, the challenge is to apply this on *in vivo* analyses of tissue myeloid cells, adding the complexity of the tissue environment, where several triggers occur at the same time, as well as some factors being produced constitutively by stromal cells (126, 127). Today, the micro environmental-induced transition of function in monocyte-derived cells is rather considered as a spectrum, and the same cell can undertake different functions over time depending on the situation (128-130). The transition is thought to induce epigenetic changes, which can explain tolerance and the “trained immunity”, e.g., the fact that a second LPS stimulation not yields the same strong response as the former (131, 132). The knowledge that different stimuli change the phenotype and transcriptional as well as epigenetic programming of the monocyte-derived macrophages and tissue-resident macrophages identifies them as potential candidates in cell-based

therapies to restore homeostasis and dampen T-cell responses (133, 134). Taken together, it is important to assess the environment of interest in order to link a certain cellular phenotype and its function. When *in vitro* studies are conducted it is of relevance to utilize the proper combination of stimuli mimicking the tissue or disease of interest, which we have tried to adopt in our research by the use of three-dimensional (3D) tissue models of oral and lung mucosa, allowing the differentiation and polarization of monocytes and monocyte-derived cells to occur in a tissue-like environment (135-137).



Figure 7. An illustration on the spectrum of monocyte and macrophage functions, highlighting their importance in tissue homeostasis and host defence. *Illustration by Sofia Björnfor Holmström.*

1.3.3 Tissue-resident macrophages

In several locations throughout the body the tissue-resident macrophages originate from an embryonic progenitor and self-renew in adulthood without being replenished by monocyte-derived cells (138). To properly distinguish the different subsets of the mononuclear phagocytes it is of importance to understand their ontogeny (128, 139). Considering the shared functions and phenotypic markers between the tissue-resident macrophages and the adult monocyte-derived macrophages, understanding their origin might enable to track specific identities of the distinct subsets. To address this issue, several fate-mapping studies have been conducted in animal models. These studies have revealed that the tissue-resident

macrophages of liver, epidermis, brain, lung and kidney, namely the Kupffer cells, Langerhans cells, microglia, alveolar macrophages and the resident kidney macrophages, all develop at an embryonic stage independently from the adult hematopoietic stem cells in steady state conditions (48, 138, 140, 141). Another study also show that the erythro-myeloid progenitors (EMPs) migrate from the yolk sac into the fetal liver to become macrophage precursor, followed by a CX₃CR1-dependent migration into fetal organs, where they undertake different transcriptional programs as part of the organogenesis (142). Though, Karjalainen and colleagues showed that it is only the brain microglia and partially the epidermal Langerhans cells that originate from the yolk sac precursors, while the other derives from fetal hematopoietic progenitors via fetal monocytes (143). In line with this, CX₃CR1 is mainly expressed by the microglia among the different tissue-resident macrophages (129). There are also studies supportive of this in humans, where patients with a GATA-2 mutation lack monocytes, DCs and NK cells, however they have alveolar macrophages and Langerhans cells (144). In line with this, the Langerhans cells in the epidermis remain as long as 10 years after transplantations in humans (145, 146). The early establishment of embryonic macrophages in all organs indicates their importance in tissue development and homeostasis. Well in place resident macrophages in distinct tissue environments acquire epigenetic modifications to gain tissue-specialized functions (147).

The survival and proliferation of the tissue-resident macrophages are dependent on the CSF1R signaling, however CSF1 knock out mice still retain a proportion of the microglial and Langerhans cells, though a second recently discovered ligand, IL-34, is suggested to be important for the survival of the resident macrophages in the brain and epidermis (148-150). There is also evidence that there is a role for CSF2 in the longevity of the resident alveolar and intestinal macrophages (151, 152). In the context of inflammation, infection or injury to the tissue, adult bone marrow-derived monocytes can enter all tissues including brain to become macrophages (153). In addition, the monocyte-derived cells also replenish the embryonically originated tissue-resident macrophages in the gut, cardiac tissue, and partly in the dermis (154-156). In line with this, an elegant study of the peritoneal cavity showed that the monocyte-derived macrophages stay at least for several months after inflammation or infection to support the tissue-resident macrophages in shaping the adaptive immunity (12).

An important function of the tissue-resident macrophages besides organ development and inflammation is the efferocytosis of neutrophils that shifts the phenotype of the macrophages into an immunosuppressive phenotype, with production of IL-10 and growth factors such as transforming growth factor (TGF)- β , and VEGF (157). In addition, macrophages can induce the production of TNF-related apoptosis-inducing ligand (TRAIL) that induce neutrophil apoptosis followed by their subsequent up-take by macrophages, and has been suggested as a therapy in chronic inflammatory diseases (158). The efferocytosis is thought to be an important mechanism in the maintenance of tissue homeostasis (12).

Reviewing these articles shed light on the difficulty to identify specific markers for the different sources of tissue macrophages when analyzing human tissue samples, though

CCR2, CD14 and TREM-2 might be candidate markers to identify adult monocytes and monocyte-derived macrophages, and the addition of MerTK could potentially separate the latter two (129, 156, 159, 160). The above described environment-induced transition into a spectrum of functions also account for the tissue-resident macrophages. Recent advances in the research on induced-Pluripotent-Stem-Cells (iPSCs) have discovered a way to obtain primitive macrophages (iMacs), which further differentiate into tissue-resident macrophages (161). These cells might be help-full in the understanding of the tissue-resident macrophages. Addressing functions of the tissue-resident macrophages under specific conditions is of importance to increase the understanding of their role in disease development.

1.4 MONOCYTES IN CHRONIC INFLAMMATORY DISEASES

In this section, I have highlighted the current knowledge on the involvement of monocytes and monocyte-derived cells in chronic inflammatory diseases, with particular focus on the commonly occurring disease Periodontitis (PD), as well as the rare disorder Langerhans cell histiocytosis (LCH). Even though these diseases are different they share some features such as the involvement of myeloid cells and bone manifestations, as well as the increased levels of MMPs and the cytokine IL-17A that are associated to both PD and LCH pathogenesis (162-164). Also, LCH can present with periodontal manifestations, linking the pathogenesis of these diseases (165, 166).

1.4.1 Periodontitis

1.4.1.1 The gingival mucosa

The oral cavity is a part of the digestive machinery and also in close connection to the respiratory tract. The majority of the oral cavity barriers are covered by a non-keratinized squamous epithelium, called lining mucosa. Covering the alveolar bone is the masticatory mucosa, named gingiva, which is a stratified squamous epithelium with varying degree of keratinization. Keratinization is the differentiation process of the epithelial cells, starting in the stratum basale where the proliferation occurs, followed by a differentiation along the stratum spinosum and stratum granulosum (167). The outer layer of the keratinized epithelium is non-vital and called stratum corneum and can only be found in parts of the gingiva that are exposed to mastication. Other cells in the oral epithelium are the Langerhans cells and intraepithelial lymphocytes (IEL), which are important for the control of the barrier defense through communication with the epithelial cells (168, 169). A basement membrane separates the epithelium from the highly-vascularized lamina propria, harboring a wide array of immune cells (170-172). The gingival mucosa that is in contact to the tooth is divided into the sulcular epithelium (SE), which is not attached to but surrounds the tooth, and the thin permeable junctional epithelium (JE) that is the gingival attachment to the tooth or root (Figure 8). Both the SE and JE are non-keratinized and therefore more vulnerable to insults from the commensals as well as pathogens and other triggering factors. Immune cells and

antimicrobial factors are constantly released through the JE into the gingival cervical fluid (GCF), as a part of the defense against pathogens and the control of the commensals (173).

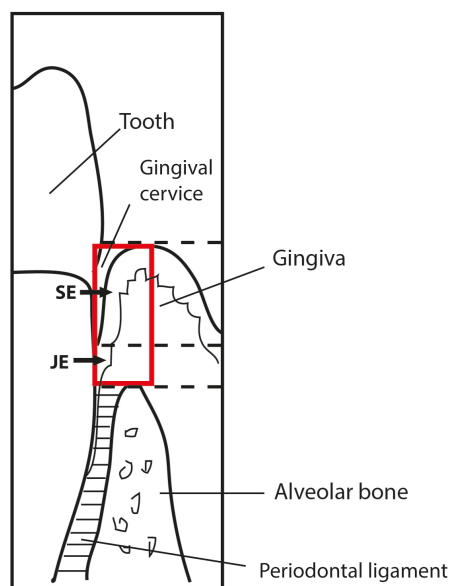


Figure 8. Illustration of the tooth and its supportive structures. The epithelium lining towards the tooth is divided (dashed line) into the sulcular epithelium (SE) and the junctional epithelium (JE). The space between the SE and the tooth is called the gingival cervice, and holds the gingival cervical fluid (GCF). The distance between the upper and middle dashed lines can be measured with a graded periodontal probe, and is referred to as the periodontal probing depth.

Illustrated by Sofia Björnfot Holmström.

1.4.1.2 Periodontal diseases

Gingivitis is a reversible inflammatory reaction towards dental plaque accumulation, diagnosed by bleeding on probing (BOP) (174). Gingivitis is thought to be a stable protective mechanism by the host to control the microbiota, and breaches in homeostasis and transition into the destructive disease PD only appears in a part of the population (175, 176).

Periodontitis (PD) is a multifactorial chronic inflammatory disease initiated in the gingival mucosa, leading to subsequent destruction of the neighboring tooth supportive structures (177-179). In PD, the inflammatory reaction results in a migration of the JE along the root towards the apex, leading to an increased length of the SE (180, 181). The underlying process is probably due to protease-mediated degradation of the collagen fibers under the JE (182, 183). The process will lead to deeper gingival pocket and a subsequent increase in the bacterial load. The cause of periodontitis is speculated to be a failure of the host immune cells to maintain homeostasis with the commensal microbiota in the gingival cervices, leading to subsequent host-mediated immunopathology (184, 185). A systematic review identified the global prevalence of severe PD to 11.2 % (186). It is speculated that the tip-over from gingivitis into PD occur due to a skewed microbiota, known as dysbiosis, allowing certain commensals to increase and become pathobionts (187, 188). Lately, an important question has arisen within the PD research field: “Do the bacteria select the disease or does the disease select the bacteria?” and the authors behind the question suggests the latter (33). The transition of gingivitis into PD is rather considered to be host mediated (33, 185), and can be due to several factors such as immunoregulatory defects, epigenetic changes, immunodeficiencies or systemic diseases, medication, hormonal changes, smoking, diet and stress, leading to aberrant inflammatory responses (189-195). The inflammatory environment

and the tissue degradation results in nutrition and selection for asaccharolytic gram-negative bacteria, which have been associated with PD pathogenesis, though they are now rather suggested to be opportunistic than causative (33, 196). Blocking inflammatory pathways also restore the dysbiosis, further strengthening the theory that the disease selects the bacteria (197). The change in the microbiota due to environmental changes is called the ecological plaque hypothesis, and so far no specific disease-causing PD pathogens have been identified, but rather several pathobionts that contribute to the disease progression (33). This indicates that it is important to shift the focus from the microbiota to the host mediated responses and introduce treatments that control the inflammation. In line with this, treatments targeting host inflammatory pathways, especially the resolution of inflammation, have been introduced with promising results in animal studies (197-199). There are also modulatory treatments introduced in human PD in conjunction to mechanical plaque control, such as the sub-antimicrobial low dose doxycycline that inhibits MMP activity and therefore reduce the tissue degradation (200). Also, systemic treatment with aspirin or the cyclooxygenase-2 (COX2) inhibitor Celecoxib, showed promising results in the conjunctive treatment of PD (201-204).

In order to understand the host mediated aberrant immune responses, it is important to characterize the cells and cellular mediators that give rise to the uncontrolled inflammatory reactions and tissue degradation. In addition, it is central to understand the process of tissue homeostasis in the gingiva. Given the mechanical injuries from mastication and oral hygiene routines and the continuous communication with the microbiota, it is impressive that the host manages to maintain barrier functions and tissue homeostasis. In order to maintain homeostasis, the immune cell compartment in the gingiva needs to balance inflammatory reactions with mechanisms for tolerance and wound healing. The commensals are suggested to be involved in this process in the intestine and skin via their interplay with the epithelium and the immune cells, but little is known about this process in the gingival barrier (18, 205-208). This was highlighted in an elegant review recently published by Moutsopoulos and Konkel (209). Though it is suggested that the oral commensals induce the production of an inflammatory response to protect against pathogens, while a dysbiosis of the microbiota results in an immune evasion mechanism (210-212).

Interestingly, mastication is also shown to be important for tissue homeostasis via inducing the accumulation of T_H17 cells, which produces cytokines like IL-22 that are important for barrier integrity (213-215). Nevertheless, the T_H17 cells are also implicated in the pathogenesis of PD via their increased production of IL-17A that triggers inflammatory reactions and osteoclastogenesis (216). Moving a step backwards, the epithelial cells, fibroblasts and myeloid cells in the tissue are important for the initiation of the inflammatory reactions and produces cytokines like IL-23 that activates the T cells, innate invariant lymphocytes, ILCs, and the myeloid cells themselves (217). Strengthening this, IL-23 and IL-17A are both associated with PD, and are implicated as treatment targets in other destructive inflammatory diseases (218, 219). In addition to IL-23, the PD innate cellular

responses also include other cytokines and chemokines, as well as MMPs that together contribute to tissue inflammation and degradation (183, 220-222).

The neutrophils are frequently found in the GCF in “health” and further increased in PD, suggesting that they are involved both in the maintenance of tissue homeostasis and in the escalated inflammatory reactions in PD (223). In line with this, a knock out mouse model in Del-1, an inhibitor of LFA-1-mediated neutrophil recruitment, resulted in aggravation of PD (224). In genetic disorders where the neutrophils are defect, early-onset periodontitis is often occurring, showing their importance in the control of the commensals as well as their importance in the resolution of the inflammation, where the efferocytosis of neutrophils by tissue-resident macrophages reprograms the macrophages into anti-inflammatory effectors producing IL-10 and TGF- β (225-228). Microbial stimuli can induce epigenetic changes in the myeloid cells that leads to a trained immunity/tolerance, rendering them less responsive to a second encounter, though this mechanism can also be utilized by pathobionts to escape killing by the host (132, 208). Epigenetic changes can also render the cells hyper-responsive, which is shown for the oral epithelial cells upon TLR2 stimulation (229).

Based on studies in a peritonitis mouse model, the infiltration of monocytes in the post-resolution phase of inflammation is also suggested to be important in the shaping of the adaptive immunity with an increase in suppressive T_{reg} cells and the establishment of a primed homeostasis that dictates subsequent reactions (12). This may also be carried out by the tissue-resident macrophages at steady state conditions and in addition they migrate to lymph nodes to suppress the adaptive immune response in an iNOS-dependent manner (12). In a mouse model, ablation of the resident Langerhans cells increased the inflammatory driven alveolar bone loss, suggesting their importance as immune-suppressors (230, 231), though this needs to be further investigated in human gingival tissue. The fact that the gingival barrier constantly suffers from injuries, tissue repair and wound healing are important functions required. These functions are potentially carried out by the monocyte-derived macrophages and the tissue-resident macrophages in the gingiva, given their ability to transition into a remodeling and wound healing phenotype with production of ECM components and growth factors (75, 208).

In summary, the T_H17 cells and neutrophils are central players in PD pathogenesis, though I propose to move the spotlight on the myeloid mononuclear cells. They are likely to be involved in all steps of the inflammatory reaction, including the initiation, progression, resolution and post-resolution, and therefore potential immunomodulatory targets. Though we need to increase our understanding on their functions in gingival tissue as well as their role in human PD, in order to identify potential pathways to modulate the destructive inflammatory reaction.

1.4.1.3 Monocytes in blood, and monocyte-derived and tissue-resident macrophages in gingival tissue in PD

The presence of different myeloid cells in gingival tissue in health and PD, are beginning to be characterized, though their origin as well as their phenotypes and functional roles remain to be further elucidated. In common with skin, the gingiva also holds Langerhans cells (HLA-DR⁺CD1a⁺Langerin⁺) in the epithelial layer (146, 148, 232-234). The resident macrophages in the lamina propria are speculated to consist at least partly of adult bone marrow-derived monocytes that differentiate into monocyte-derived macrophages, as in lamina propria of the intestine (84). In line with this, the monocyte chemoattractant CCL2 is expressed in gingival cervicular fluid that is known to reflect the gingival environment, and its levels are increased in PD (235, 236). The monocytes/macrophages in the lamina propria express HLA-DR as well as CD68, which expression is not detectable in the epithelial compartment where the tissue-resident Langerhans cells reside (Figure 9).

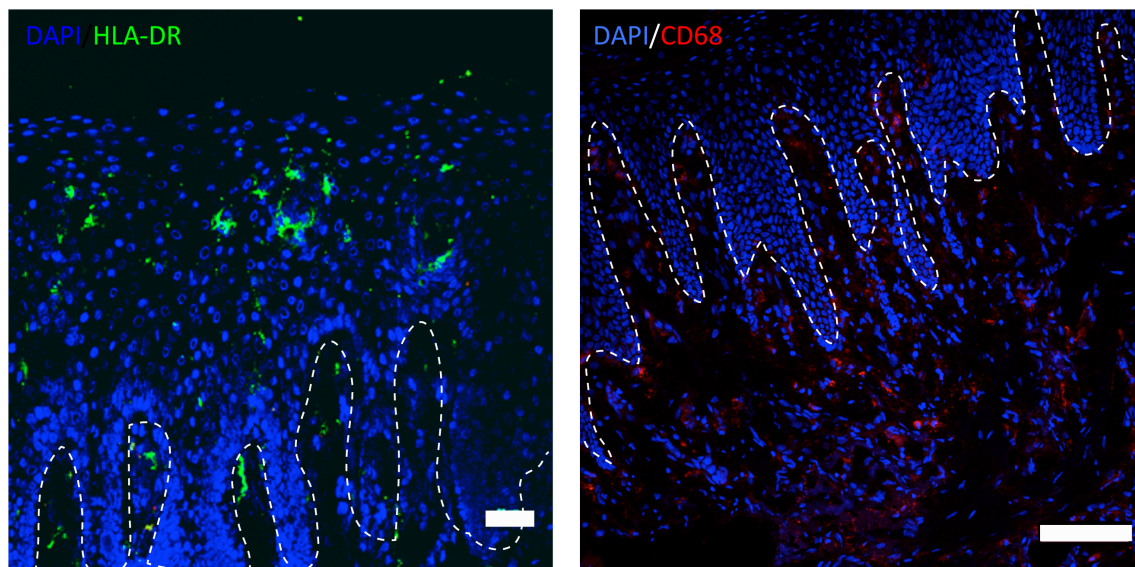


Figure 9. Confocal images of gingival tissue biopsies showing nuclei staining (blue, DAPI), HLA-DR (green, left) and CD68 (red, right). The dashed lines mark the epithelial lining. The HLA-DR-positive cells include both tissue-resident intraepithelial Langerhans cells and the lamina propria macrophages (likely to be monocyte-derived), while the CD68 staining is only detected in the lamina propria. Scale bars represent 50 μ m in the left image and 100 μ m in the right image.

RNA sequencing of peripheral blood monocytes from patients with PD, has identified that monocytes from PD individuals are more prone to inflammatory reactions, such as apoptosis, cytokine production and antigen presentation (237, 238). In line with this, it is shown *in vitro* that monocytes from PD patients, as well as monocyte-derived DC, produce elevated levels of inflammatory cytokines such as IL-12 and IL-1 β and less anti-inflammatory IL-4 and IL-10 (239, 240). This suggests that PD monocytes are prone to induce amplified inflammatory reactions already in circulation. Possible explanations may be the higher incidence of bacteraemia, release of danger signals from inflamed gingival tissue into blood that can activate the monocytes, or due to an inherited altered phenotype of the monocytes in individuals that develop PD. In line with the former speculation, blood DCs are shown to

carry antigens from oral commensals in circulation (241). The total number of blood monocytes is elevated in individuals with PD and studies on the monocyte subset composition in PD reveal increased proportion of the non-classical CD14⁺CD16⁺⁺ monocytes (103, 242). In another previous study, the monocytes were grouped as one population, and the monocytes from the PD patients had an increased percentage of cells positive for CD16, which correlated with lower CD14 expression, and *in vitro* LPS stimulation recapitulated the differentiation of the monocytes into CD16-positive cells (243). An increase in the CD16-positive monocyte compartment may reflect an activation or differentiation of the monocytes, given their developmental relationship (36, 48). Maybe the increased systemic inflammation in individuals with PD also influences the production and differentiation of monocytes in the bone marrow, where only the classical monocyte phenotype is present at steady state (36). Another speculation is that the increase in proportion of the non-classical monocytes in blood might be due to the increased recruitment of CD14⁺⁺CD16⁻ monocytes into inflamed gingival tissues. The monocytes in PD induce T_H17 immune responses and the classical monocytes from PD compared to healthy individuals are more prone to differentiate into osteoclasts (217, 244, 245). Notably, the increased production of IL-17A by the T_H17 cells was reported to induce osteoclast activation, MMP production and bone degradation (162, 246). In line with increased osteoclastogenesis, the RANKL-osteoprotegerin system is a promising biomarker candidate for PD (182, 247).

Analyses of the immune cells in gingiva have mainly focused on the composition of immune cells, and revealed increased number of myeloid cells in PD (171, 172, 248). Attempts have been made to study the phenotype, where a study identified an increase in the proportion of CD16-positive monocytes/macrophages in chronic PD tissue (103). In addition, histological analysis of control and PD gingival tissue revealed that CCR7 and iNOS were more frequently expressed on the macrophages in PD, than CD163 and CD206, suggesting an inflammatory phenotype (249). Recently, human gingival tissue from PD and control individuals have also started to be explored by multi-parameter flow cytometry, which is a step towards identifying cell subsets and linking certain functions to specific cells (170). That study identified a similar proportion of the myeloid mononuclear cells in PD and controls of the total CD45-positive cells; nevertheless the CD45-positive cells displayed a 10-fold increase in PD gingiva (170). Another study analysing the gingival tissue with flow cytometry identified that the CD68⁺HLA-DR⁺ cells also expressed CD163, CD11c, TLR2 and TLR4 (217). The macrophages are important for the maintenance of tissue homeostasis, as well as in inflammatory reactions (104, 129, 250). In line with this, targeting specific macrophage subsets has shown to be beneficial in the treatment of PD in mice models (251, 252). Taken together, the monocytes/macrophages in the gingiva of PD display a proinflammatory phenotype and besides local inflammatory reactions, PD is associated with systemic changes in the blood monocyte compartment. The origin of the myeloid mononuclear cells in gingival tissue and the gingiva-specific functions of tissue-resident macrophages, monocyte effector cells, as well as the monocyte-derived cells need further investigation. To date they are mainly grouped as one macrophage population.

1.4.2 Langerhans cell histiocytosis

LCH is a rare chronic inflammatory disorder, effecting either single or multiple organs with manifestations in bone or soft tissues, such as lung and skin, as well as periodontal tissues (165, 166, 253, 254). The LCH granulomas have also been associated with activation in the mitogen-activated protein kinase (MAPK) pathway, Ras-RAF-MEK-ERK, and partly associated with a mutation in the *BRAF*^{V600E} gene that is involved in this pathway, suggesting a neoplastic etiology as well (255, 256). In line with this, the mutation has been detected in classical and non-classical monocyte as well as in CD1c⁺ blood DCs (257). Though it is not verified if the mutations occur due to the chronic inflammatory environment or if the mutation and the proliferation of LCH cells induce the inflammatory reactions seen in LCH. Langerin⁺CD1a⁺ cells, as well as multinucleated cells are signatures of the LCH lesion; therefore the resident Langerhans cells, as well as the DC has been associated with the LCH inflammatory reactions and granuloma formations (163, 258). It is now known that the LCH cells are more closely related to bone marrow-derived cells rather than the tissue-resident Langerhans cells, and are suggested to derive from monocytes in a Notch1-DLL1 dependent manner, as well as from subsets of DCs (258-260).

Monocyte-derived DCs as well as LCH lesion cells have been shown to express the IL-17A receptor (IL-17RA) and to produce IL-17A, a cytokine potent of inducing granuloma formations and bone manifestations, via the induction of several other cytokines and MMPs (163, 261). Monocyte-derived DCs responded to IL-17A with the formation of multinucleated giant cells and the production of MMPs and tartrate-resistant acid phosphatase (TRAP), in a similar way as when differentiating monocytes to osteoclasts using CSF1 and RANKL (163). Both MMP9 and MMP12, which were produced by the multinucleated cells, have been detected in LCH lesions (262, 263). In LCH patients, high levels of proinflammatory cytokines are present already in blood, though little is known about the contribution from the circulating monocyte in LCH, which are lately assumed to be the source of the LCH cells (257). Increasing the understanding of the myeloid blood cells in LCH is therefore important to better understand the disease on-set and to identify new ways to treat LCH. In line with this, we have analyzed the blood monocytes in LCH, and identified them as producer of IL-17A in circulation, which is not a normal occurrence in blood monocytes (164). This has paved the way for a new treatment of LCH, in depleting the monocytes by apheresis, which reduced the pathological IL-17A levels (264).

1.4.3 Other chronic inflammatory diseases associated with PD

1.4.3.1 Cardiovascular disease

The increased risk for cardiovascular disease (CVD) in individuals with PD is well accepted and speculated to be due to the presence of periodontal microbes in the circulation as well as the increased systemic inflammation and activated immune cells that is associated with PD (265, 266). The systemic inflammation in PD leads to altered endothelial cell functions, with increased activation, whereas periodontal treatment improved the endothelial function (267).

In common with PD and other chronic inflammatory diseases, the monocyte subset composition is altered in CVD, with different studies showing an increase in either the intermediate or the non-classical monocytes (268, 269). Another study identified elevated numbers of the classical monocytes as predictor of cardiovascular disease (270). Atherosclerosis is a common chronic form of CVD that can lead to thrombosis, myocardial infarction and stroke if the atherosclerotic plaque is ruptured (271). Macrophages are the main immune cell component in the plaque and are suggested to differentiate from recruited blood monocytes (272). The migration of monocytes into the lesion is suggested to depend on CCR5, and a role for CCR2 is also suggested (273). The engulfment of cholesterol by the monocyte-derived macrophages leads to the formation of foamy cells, which mature and form the plaque (274). The monocytes and macrophages are able of producing inflammatory cytokines and MMPs that contribute to the thinner wall of the plaque and an increased risk of rupture (108). In addition the MMPs generate matrikines from degraded ECM, especially from elastin, that are chemotactic for monocytes, and further increase their recruitment to the injured vascular wall (275). It is suggested that the intermediate monocytes are involved in the pathogenesis of atherosclerosis, and even though there are conflicting data on the increase of different subsets, the intermediate monocytes are the most frequently reported subset to increase in the circulation of patients with CVD (276). In line with this, the intermediate monocytes are suggested to be a predictor of atherosclerosis and plaque rupture (64). In addition to chronic inflammatory disease, other factors such as age and ethnicity has also been shown to alter the monocyte subset composition and functions (63, 277).

1.5 THREE-DIMENSIONAL TISSUE MODELS TO STUDY MONOCYTES AND MONOCYTE-DERIVED CELLS UNDER PHYSIOLOGICALLY RELEVANT CONDITIONS

The three-dimensional (3D) modeling technique was introduced already in the end of the 19th century (278-280) and is based on the combined culture of different tissue constituent cell types in an ECM-like structure, and can be expanded to include immune cells. This technique can be employed in studies on the tissue response to infections, or symbiotic versus dysbiotic microbiota, to study tissue reconstruction after wounding, toxicology and pharmacology, stromal-immune cell interactions, tumor development, and for tissue transplant engineering, just to mention some (210, 281-286). Stimulations of monocultures can be useful in understanding certain pathways, though the *in vivo* situation is far more complex and a single stimulus never occur. In tissue, different cells respond differently to each stimulus, with the production of a wide-array of different effector molecules.

Monocytes are highly plastic cells as told, with functions ranging from pathogen defense, antigen presentation, release of immunomodulatory cytokines, as well as the differentiation into monocyte-derived cells resembling and potentially replenishing resident DCs, macrophages and osteoclasts in several tissues (12, 36, 45, 100, 101, 111). It is challenging to study the fate of the monocytes in different tissues over time in humans; therefore the

knowledge to date on monocyte tissue-functions is largely based on animal models. However several responses are species specific, and the fact that the mouse monocytes are composed of two subsets, compared to the three described subsets in humans, further motivate the development of human tissue models with monocytes. Therefore, we chose to develop reproducible *in vitro* 3D model systems of human oral and lung mucosa for the purpose of studying phenotypic and functional changes of monocytes and monocyte-derived cells in tissue inflammation. Given their wide array of important functions in the maintenance of tissue homeostasis as well as in the orchestration of inflammatory reactions, the monocytes and their derived cells are potential targets in the development of immunomodulatory strategies to manage or halt chronic inflammatory diseases (287).

2 AIMS

The overall aim of this thesis was to increase the understanding on how monocytes can contribute to the development and progression of chronic inflammatory diseases, with the main focus on periodontitis (PD).

Specific aims:

- Establish methods to explore monocyte differentiation and function within inflamed tissues, by utilizing and further developing our 3D tissue models
- To study the gingival tissue signature in PD with regards to monocyte/macrophage functions, and specifically their contribution to tissue degradation
- Assess potential monocyte-associated salivary biomarkers for PD
- To explore the phenotype and functions of the monocytes in peripheral blood in patients with chronic inflammatory diseases

3 STUDY DESIGN

In this section of the thesis, I give an introduction to the methodologies applied to address the specific aims. Detailed information about the methods and materials used you can find in the respective manuscripts and published articles.

3.1 ETHICAL CONSIDERATIONS

The regional ethics committee in Stockholm, Sweden, approved the collection of gingival tissue samples, and the collection of blood samples from LCH patients, PD patients and healthy controls, as well as the use of buffy-coated blood from anonymous donors. The regional ethics committee in Lund, Sweden, approved the collection of saliva samples and the clinical examinations. All the participants gave their written informed consent prior to attending a study, and all the studies were in accordance with the Declaration of Helsinki.

3.2 STUDY GROUPS AND SAMPLE COLLECTIONS

3.2.1 Gingival tissue samples

The collections of gingival tissue samples were performed in conjunction with planned surgical procedures due to periodontitis, implant surgery, or tooth extractions. In total, gingival tissue samples from 33 patients with periodontitis and 30 controls were collected. The inclusion criteria for the PD patients were ≥ 4 teeth with a probing depth of ≥ 6 mm, and a persistent gingival pocket ≥ 6 mm at the site of sample collection. The control patients had no gingival pockets > 4 mm and no history of PD (Table 1, **paper I**). Patients that had taken antibiotics or corticosteroids during the last three months prior sample collection and patients with HIV, hepatitis or diabetes were not included in the study. Gingival tissue samples for gene expression analysis were collected and stored in RNeasy lysis buffer for 1-3 days at 4°C, followed by removal of the RNeasy lysis buffer and storage in -80°C until RNA extractions. For western blot analysis, gingival tissue samples were directly placed on dry ice and stored in at least -80°C until protein extraction. Gingival tissues for histology analysis were collected in Histocon and embedded in Cryomount within 24 h and stored at -80°C. Finally, samples for flow cytometry analysis were collected in complete RPMI 1640 medium on ice and processed within 4 h of collection. Regarding the small size of the samples (3-5 mm), it was not possible to perform all the different analyses on each individual patient sample.

3.2.2 Whole blood or buffy-coated blood

Whole blood was collected in EDTA-containing vacutainers and peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep gradient centrifugation. The plasma was saved for protein analysis. Monocytes were isolated with negative selection using EasySep monocyte enrichment kit without CD16 depletion. PBMCs were stained with fluorochrome-conjugated antibodies and analyzed with flow cytometry. Monocytes were either processed directly for mRNA analysis or cultured and stimulated for subsequent protein and gene expression analysis. Monocytes isolated from buffy-coated blood, with the

same protocol as for whole blood, were subsequently used for *in vitro* differentiation and stimulations, implantations into 3D mucosa models or used for flow cytometry analysis.

3.2.3 Saliva samples

The concentration of MMP12 and the S100 proteins S100A8/A9 and S100A12 in saliva were analyzed in a previously collected cohort of samples from a total of 436 participants (288) (Table 1, **paper II**). The participants were randomly selected and invited to fill in a health questionnaire, to take part in an oral examination and to provide saliva samples. The questionnaire included questions about systemic diseases, medication, smoking and experiences of oral healthcare. A detailed clinical examination was performed on all individuals that wanted to participate in the study, and included radiographs for evaluation of alveolar bone level and caries lesions, registration of bleeding on probing (BOP), periodontal probing depths (PPD/PD), plaque index (PI), number of manifest caries lesions (MCL), and registration of decayed, missing and filled teeth (DMFT). The participants chewed on paraffin to stimulate saliva, which was collected and stored for short term at -20°C, followed by centrifugation, preparations of aliquots and storage at -80°C until protein analysis.

3.2.4 *In vitro* culturing and differentiation of monocytes

Isolated monocytes were cultured in complete RPMI 1640 media with the addition of 50 ng/ml CSF1 for a total of seven days, with half of the media being replaced at day five with new CSF1 added, to obtain monocyte-derived macrophages. To generate monocyte-derived DCs, the monocytes were cultured in the presence of 25 ng/ml CSF2 and 12.5 ng/ml IL-4. Monocyte-derived macrophages were used for determining the MMP12-inducing mechanisms, and were therefore stimulated with Prostaglandin E₂, TNF, LPS as well as CSF2. To study the role of the CD200/CD200R pathways, a CD200 Fc chimera was added to the cultures together with CSF2. Following stimulations supernatants were collected, centrifuged and subsequently analyzed for the production of MMP12 with ELISA.

3.3 THREE-DIMENSIONAL MUCOSA MODELS

To study monocytes and monocyte-derived cells in a tissue setting under steady state or inflammatory conditions, we utilized and further developed 3D tissue models of oral and lung mucosa (136, 289). The models were generated in 6-well cell culture inserts, by the coating of the membranes with a non-cellular collagen type I matrix layer, followed by a cellular collagen layer containing either primary gingival fibroblasts for the oral mucosa model or the MRC-5 lung fibroblast cell line for the lung mucosa model (Figure 10). These layers also contained concentrated Dulbecco's Modified Eagle Medium (DMEM), and after solidification of the collagen, normal complete DMEM was added to the wells in which the inserts were submerged. During a period of seven days fibroblast remodeled the collagen and cultures contracted, as the medium was changed every second to third day. This resulted in the formation of a crater-like structure, on which the monocytes or monocyte-derived DC (CSF2 and IL-4 differentiation), were seeded, and allowed to attach for one day (Figure 10).

Then the TERT-2 immortalized normal oral keratinocyte line (OKF6/TERT-2) (290) or the normal bronchial epithelial cell line (16HBE), immortalized with SV40 large T antigen, was added on top of the stromal matrix layer. The models were submerged in media for another two to three days, followed by culturing in the air-liquid interface, which allowed stratification of the epithelium (Figure 10). This set-up yielded reproducible model systems that we could use to study and modulate the myeloid cells in a tissue context.

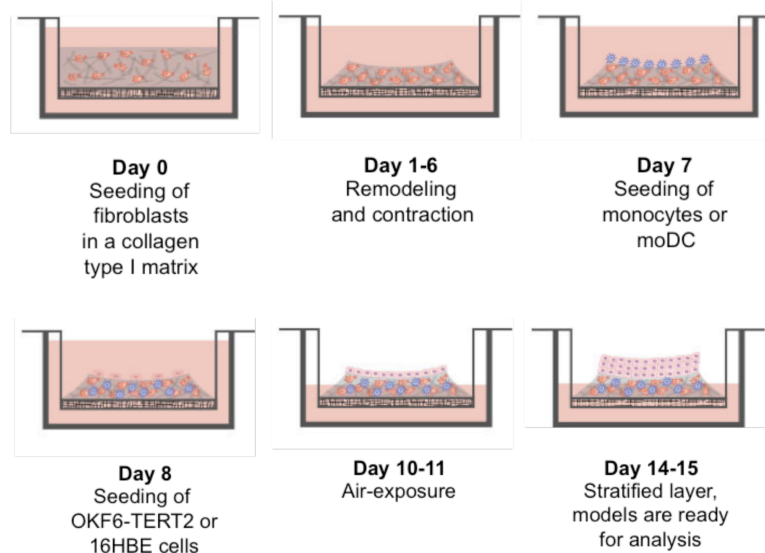


Figure 10. Oral and lung mucosa model set up. Primary gingival fibroblasts (oral) or MRC-5 lung fibroblasts are expanded in culture flasks, then harvested and resuspended in a collagen type I matrix that is added into cell culture inserts on Day 0. The cultures are submerged in medium for seven days allowing fibroblasts to remodel the collagen and form the extra cellular matrix. Subsequently, primary monocytes or CSF2 and IL-4 differentiated monocyte-derived DCs (moDC) are added on top of the stromal matrix, the “lamina propria”. At day 8, the epithelial cells are harvested from culture flasks, seeded onto the lamina propria, and allowed to form a monolayer submerged in media. After two till three days the co-cultures are air-exposed, and cultured like that for additional days to allow epithelial cell differentiation and generation of a stratified epithelium. Inflammatory stimuli were either introduced at day 9 and with a refresh in conjunction to medium changes, or a single stimulus for the last 18-24 h of the culturing period. *Illustrated by Puran Chen.*

Analyses of monocytes differentiating in the oral mucosa models were performed with flow cytometry, in parallel with *in vitro* differentiation of monocytes with CSF1 or with CSF2 and IL-4; to resemble macrophage-like or DC-like phenotypes of the monocyte-derived cells. To determine the effect of tissue inflammation on monocyte differentiation and function in tissue, models were repeatedly stimulated and subsequently analyzed with flow cytometry, and gene expression analysis. Culture supernatants were analyzed with enzyme-linked immunosorbent assays (ELISA) for evaluation of protein production. To study the influence of stimulation on monocyte migration in tissue as well as to target pathways of monocyte regulation, we also performed single short (18-24 hours) stimulations at the end of the culturing period. Using the lung mucosa models, we have established a live-imaging technique to track the migration and location of the monocyte-derived DCs in the lung mucosa model over time with confocal microscopy (**paper III**). A particular focus has been

on understanding the phenotype and tissue damaging potential of the monocyte-derived cells in the oral mucosa models, with a specific focus on MMP12 (**paper I**).

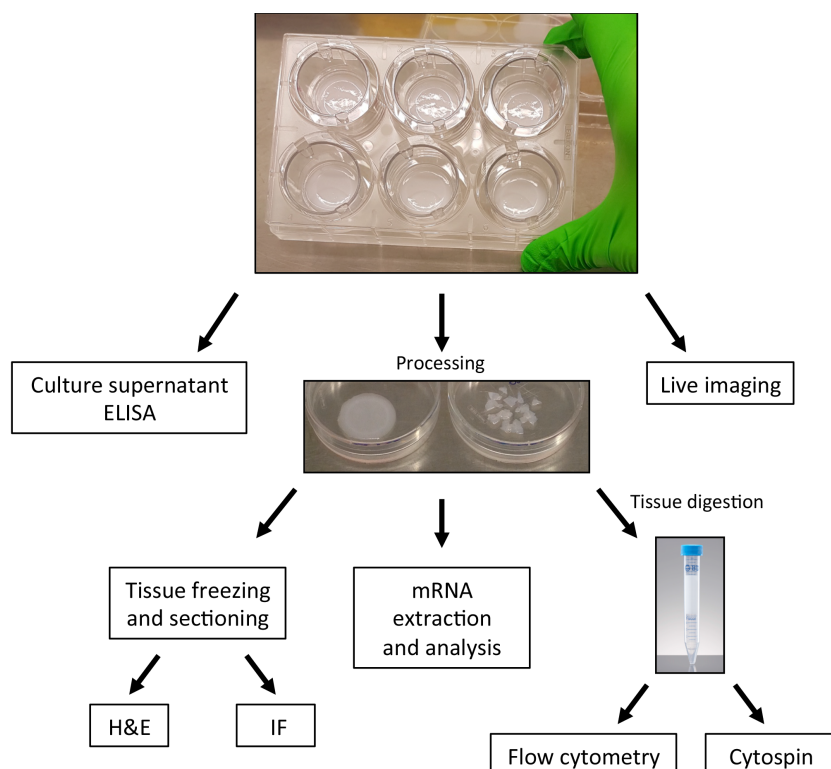


Figure 11. Schematic illustrations on the methods applied to process and analyze the mucosa models. ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IF, immunofluorescence.

3.4 GENE EXPRESSION ANALYSIS

For the gene expression analysis, RNA was extracted from the gingival tissue samples, mucosa models, and cells with different isolation methods. The RNA concentration was measured followed by a reversed transcription into complementary DNA, which was used as the template for multiplex real-time polymerase chain reaction (PCR) and the standard real-time quantitative reverse transcription PCR (qRT-PCR) analyses.

3.4.1 Multiplex real-time PCR

To address the effect of the tissue environment on monocyte-derived cells we applied the multiplex assay, analyzing the expression and relation of 12 macrophage-associated genes, including *TNF*, *CXCL11*, *PTGS2/COX2* (associated with inflammation), *IL1Ra*, *IDO*, *IL10* (immunoregulation), *DC-SIGN/CD209*, *MRC1/CD206* (scavenging) and *FNI*, *PDGFD*, *MMP12* and *TIMP1* (remodeling). This method was used to analyze the gene expression in gingival tissue samples as well as in oral mucosa models, allowing us to determine the state of inflammation in PD, as well as to compare results from the oral mucosa model with real gingival tissue. With the multiplex assay, we got the copy number of each gene reflecting the relative abundance. Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 was used as a reference gene in the multiplex assays.

3.4.2 Real-time qRT-PCR

This method was employed to determine the effect of different stimulations on cells as well as mucosa models, on single gene levels. We used the Taqman method with specific pre-made gene expression assay, which uses double conjugated probes, including both a reporter dye and a quencher, and when the probe is cleaved by the Taq DNA polymerase the dye is unconstrained and a signal can be detected (291). The number of cycles needed to give a detectable amplification/signal is measured, referred to as the threshold. The relative amount of the gene was calculated with the comparative threshold cycle method, using the formula $2^{-\Delta\Delta CT}$. Each analyzed gene was normalized to the sample expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH).

3.5 PROTEIN ANALYSIS

The gene expression analyses are screening tools that gives us an idea of what pathways that are differently regulated, though an increase in gene expression do not always relate to a changed protein production. Therefore, we also analyzed the production of the proteins of interest.

3.5.1 Enzyme-linked immunosorbent assay, ELISA

ELISA was applied to measure specific protein concentrations in liquids, such as cell culture supernatants and saliva. We used commercially available ELISA kits according to the manufacturers' instructions. The ELISAs were based on the sandwich-principle where the samples are incubated on plates pre-coated with capture antibodies selecting for the protein of interest. Following incubation, a detection antibody for the protein of interest was added to the plates and allowed to bind during a second incubation time. Subsequently unbound antibodies are washed away and the plates were incubated with an enzyme-linked antibody. Then a substrate is added and enzymatically converted into a colored product, where the intensity in coloring reflects the amount of protein in the samples. A spectrophotometer was used to determine the concentration of the proteins by measuring the absorbance. To translate the absorbance to a concentration, a standard curve with known concentrations of the specific protein is used. Saliva is challenging to analyze due to its viscosity, and therefore we performed spike-recovery and dilution tests to determine the optimal dilution of the samples, prior to analyzing all samples.

3.5.2 Western blot, WB

To determine the relative protein expression within tissues or cells, we applied WB. Prior to the WB it is important to determine the protein concentration in the lysates, to ensure that equal amount of protein is loaded, and as a control we always stained the blot with an antibody against the house keeping protein β -actin. The intensity of the protein of interest was normalized to the β -actin intensity of that sample. The WB technique mainly allows the comparison of the samples loaded on the same blot. A benefit with WB is that you can identify different forms/sizes of the protein of interest.

3.5.3 Flow cytometry

Multicolor flow cytometry allows the analysis of specific proteins on the surface or inside of individual cells. Tissue cells can also be analyzed by flow cytometry following digestions of tissue in collagenase and passing through a filter membrane, which gives a single cell suspension. The cell suspensions are labeled with antibodies that are fluorochrome-conjugated and specifically bind to cell surface or intracellular antigens. The higher concentration of antigen, the more antibody-fluorochrome complexes bind, and a stronger intensity is detected. The principle of flow cytometry is that different lasers illuminate the single stream of cells and when a fluorochrome is excited it emits light at a certain wavelength. Different filters allow the separation of multiple fluorochrome emissions that are acquired by a detector and converted into digital signals. Though the more colors used the greater challenge to properly compensate between the emissions. Flow cytometry also allow the discrimination of cells with different sizes and granularity, based on their forward or side scatter patterns. Flow cytometry can provide a lot of information on a single cell level and provides the opportunity to identify subsets of cells based on a combination of markers.

3.5.4 Immunofluorescence analysis with confocal microscopy

Confocal microscopy can provide information on the spatial distribution of certain cells or proteins within tissues, as well as their eventual co-localization. Cryopreserved tissues are subsequently sectioned, fixed and permeabilised, followed by staining procedures. Several markers can be analyzed in the same sample or section and the principle reminds of flow cytometry, where specific antibodies bind to the antigen of interest. Though in immunofluorescence (IF) analysis, signals need to be amplified, and therefore secondary antibodies conjugated with the fluorescent dye are used to detect the primary antibodies that bind the antigen of interest. We applied IF to study the location of the myeloid mononuclear cells in tissues, their co-localization with certain proteins of interest and the expression of structural proteins, where the oral mucosa models were compared with real gingival tissue. We also analyzed the effect of stimuli on the expression of ECM components. In addition, confocal microscopy enables spatial and temporal studies, referred to as live imaging analyses. We have set up a protocol to analyze the lung mucosa models in 4D (x, y, z and time) with confocal microscopy (135). The protocol used to prepare the models for the live imaging analysis is illustrated in **Figure 1, paper III**. To analyze the different cellular components in the live tissue, they had to be transduced with a fluorescent protein or incubated with a cell tracker dye prior to inclusion in the tissue models. Stimulations were added to the models in close proximity to the start of the imaging, to trace cellular migration in response to stimuli. We used a Nikon AR1 confocal laser microscope with a resonant scanner that allowed for high-speed imaging. An incubator that maintains constant temperature and CO₂ levels, to promote cell survival, surrounds the microscope.

4 RESULTS AND DISCUSSION

4.1 *IN VITRO* MODELLING OF HUMAN TISSUE INFLAMMATION

There are several benefits with *in vitro* generated 3D tissue models, including the numerous cell-cell and cell-ECM contacts that are allowed to form as well as the possibility to study mechanisms of for example immune cell fate and behavior in a more biologically relevant milieu. The first aim of my thesis work was to establish methods to explore human monocyte differentiation and function within inflamed tissues. This was conducted by utilizing and further developing our 3D models of mucosal tissues, and by introducing advanced techniques to analyze the models, including gene expression analysis, multi-color flow cytometry and live imaging.

4.1.1 Establishment of the oral mucosa model with primary monocytes

To create a reproducible system, with the monocytes as a variable, we used the normal human oral keratinocyte cell line, OKF6/TERT-2 (289, 290), which generated a stratified squamous epithelium in the model setting (Figure 12). The lamina propria was generated with human primary gingival fibroblast. We chose to use collagen I, which is the major constituent of gingival mucosa, as a supporting matrix to embed the fibroblasts in. The fibroblasts remodeled the matrix and produced other ECM proteins necessary for tissue structure and function. The addition of the epithelial cells allowed the formation of a basement membrane; expressing proteins such as laminin-5 and fibronectin (Figure 12; Fig. 4B, **paper I**), while the production of collagen-IV require a longer culturing period (280). The stratified epithelium showed a similar phenotype as real gingival tissue with the expression of the tight junction proteins e-cadherin and claudin, as well as the intermediate filament cytokeratin 16 (Fig 4B, **paper I**).

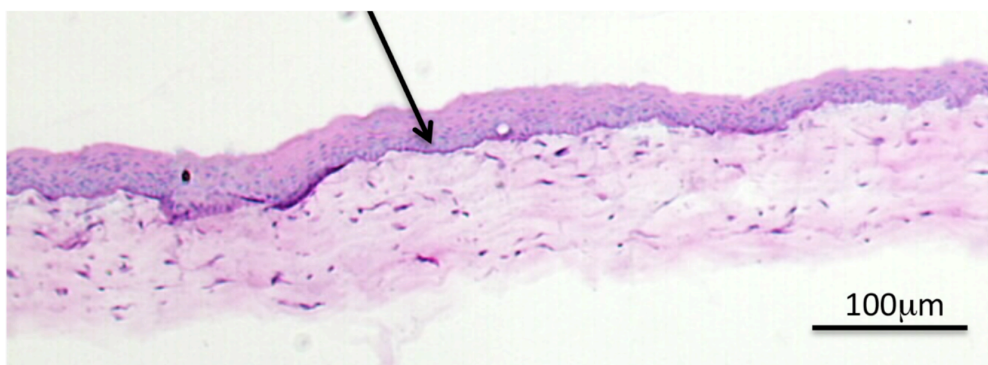


Figure 12. Light microscopy image of an oral mucosa model section stained with H&E. The image shows the formation of a stratified squamous epithelium, which is separated from the lamina propria by a more intensely stained basement membrane, indicated by the arrow

In addition to the tissue constituent cells, we also implanted freshly isolated monocytes, which did not interfere with the structural organization of the tissue models under steady state conditions. The monocytes were mainly found in the lamina propria in close proximity to the basement membrane (Fig. 4B, **paper I**). Performing the work on developing an oral mucosa

model with monocytes, we were inspired by a previously described model based on oral fibroblast and keratinocytes but no immune cells (289), and our previously described lung tissue model with immune cells (136). To the best of our knowledge, our newly developed oral mucosa model with human primary monocytes is the first such 3D tissue model to be described. Around the same time, however, another group have developed a somewhat similar model with the monocytic cell line THP-1, and investigated the crosstalk between the tissue and a 11 bacterial-species biofilm in a perfusion chamber (292). In addition to the creation of 3D models, gingival explants have also been used to study tissue-resident Langerhans cells (234). The development of an oral mucosa model with primary monocytes, we believe, is a step forward in order to increase our knowledge of human monocytes and their phenotype and function in oral tissue. This also provides an opportunity to introduce monocytes that come not only from healthy individuals, but also from individuals with certain disease states.

4.1.2 The influence of the oral mucosa on monocyte differentiation

In the 3D oral mucosa model experiments, monocyte survival and differentiation was supported by the tissue itself rather than by the addition of exogenous growth factors. Monocyte differentiation was assessed by enzymatic digestion of tissue models and subsequent analysis by flow cytometry. This revealed that the tissue model supported monocytes to differentiate into macrophage-like cells, with increased expression of CD68, CD14 and CD163, and low expression of CD209 and CD1a (Fig. 4E and suppl. figure 2, **paper I**). This phenotype resembled that of CSF1-differentiated monocytes, rather than CSF2 and IL-4 differentiated monocytes, which strongly up-regulated CD209 and CD1a, that are associated with DCs and implicated in antigen presentation (293, 294). The tissue-supported differentiation of monocytes into macrophage-like cell has also been shown in co-culture models of intestinal mucosa, alveolar epithelium and dermis (295-297). In line with the generation of a macrophage-like phenotype, the oral mucosa model constitutively produces CSF1 (Fig. 4C, **paper I**), which is important for the survival and differentiation of monocytes in tissue (58, 150). In contrast, the differentiation of monocytes into DCs might require additional constituents, such as endothelial cells, T cells, and microbial stimuli that trigger TLRs (298-300).

In the lung mucosa model experiments (**paper III**), we differentiated the monocytes into DC-like cells with CSF2 and IL-4 prior implantation, and the tissue further contributed to their longevity after implantation (136). Under these conditions, monocyte-derived DCs were found to survive up to at least 11 days in the lung model (136). The markers used to determine DC- and macrophage-like phenotypes were also employed in another recent study comparing the differentiation potential of the distinct monocyte subsets. The study showed that all monocyte subsets differentiated into macrophage-like cells while only the classical monocytes were able to obtain a DC-like phenotype and function (45). An important follow up study would be to sort and implant the three distinct human monocyte subsets into

different 3D mucosa models, to study the fate of each subset under homeostatic conditions as well as in an inflammatory setting.

4.1.3 Establishment of tissue inflammation in the mucosa models

As one of the aims with my thesis work was to dissect monocyte differentiation and functions in inflamed tissues, we introduced inflammation via the stimulation with microbial products, such as the TLR4 ligand LPS for the oral and lung mucosa models, and also the TLR2/1 ligand Pam3CSK4 for the lung mucosa models. In the oral mucosa model, LPS stimuli were used alone or in combination with IFN γ , which is produced by lymphoid cells, such as NK-cells and effector T_H1 cells in response to inflammation, and potentiate macrophage effector functions (130). The addition of IFN γ is often used in combination with LPS, and has for example been used to induce an inflammatory phenotype of macrophages in a 3D tumor co-culture model (301). To resemble the *in vivo* scenario, microbial stimuli were added on the epithelial surface of the oral and lung mucosa models.

In the lung mucosa model, we showed that stimulation with both TLR4 and TLR2/1 ligands induced DC migration (Fig. 3 and 4, **paper III**). To track migration, we measured the length and speed of the cell migration, as well as the sphericity of the cells, where a prolonged structure reflects increased migratory behavior. The effect of TLR stimulation on lung tissue inflammation was confirmed by digesting the tissue models followed by flow cytometry analysis of monocyte-derived DC and by mRNA analysis of whole tissue extracts, as well as the measurement of inflammatory cytokine production in tissue culture supernatants. We concluded that the stimulation with LPS changed the phenotype of the monocyte-derived DCs, with increased expression of HLA-DR and CD86, markers of DC activation (Fig. 5D, **paper III**). Further supporting the tissue inflammation was the observed increase in mRNA expression of CXCL8 and IL-1 β , as well as the production of TNF (Fig. 5A-B, **paper III**).

Analyzing cells from digested oral mucosa models, we identified that monocyte-derived cells (CD45-positive) up-regulated CD14 and CD80 surface expression in LPS and IFN γ -stimulated models, while CD200R was adversely affected, with a significant down-regulation in response to LPS and IFN γ (Fig. 4F, **paper I**). Similar patterns in surface expression of the co-stimulatory CD80 and the co-inhibitory CD200R molecules were observed in *in vitro* stimulations of monocytes-derived macrophage-like cells (118). In response to stimulation we also assessed the expression of CD163, which was expected to decrease based on previous *in vitro* studies, where CD163 has been implicated with anti-inflammatory functions of macrophage-like cells (119). However, we did not detect altered CD163 molecule surface expression on monocyte-derived macrophage-like cells from stimulated oral mucosa models (Figure 13). Since our model system is more complex and not as well controlled as strict monolayer cultures with added cytokines, we may see differences otherwise not observed. In addition to CD163, CD14, CD80 and CD200R, we also assessed the influence of inflammatory stimuli on the expression of CD16, CD209, HLA-DR, CD141/BDCA-3 and CD1a on the monocyte-derived cells from the oral mucosa models (Figure 13). Among all

these, CD1a was the only marker that was altered, with a significant down-regulation in response to the combination of LPS and IFN γ (Figure 13). This may be in line with the properties of the tissue model to support differentiation of monocytes towards macrophage-like cells rather than DC-like cells.

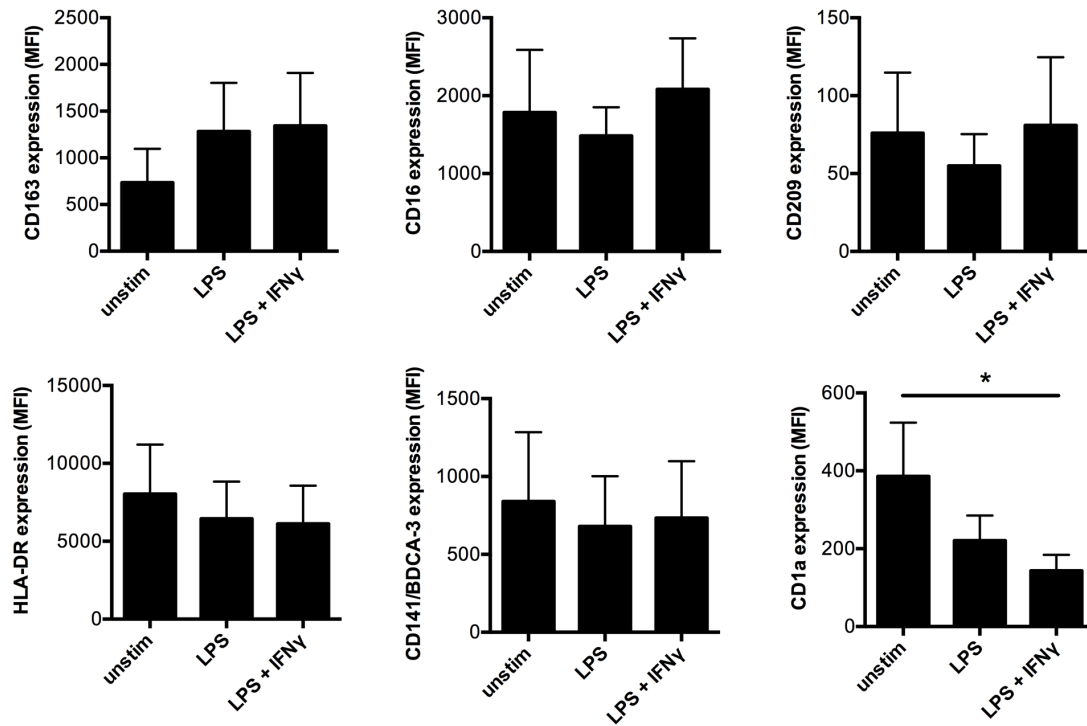


Figure 13. Flow cytometry analysis of enzymatically digested oral mucosa models after stimulations with LPS alone or in the combination with IFN γ , as indicated under the bars. The median fluorescence intensity (MFI) of each marker was analyzed on the monocyte-derived cells from four different donors. Friedman test with Dunn's multiple comparison test was applied to analyzed statistical differences, * $p < 0.05$. Bars represent mean \pm SD.

In addition to surface marker expression, we analyzed the gene expression of the un-stimulated and stimulated (LPS or LPS+IFN γ) oral mucosa models with multiplex real-time PCR, to investigate the expression of 12 different macrophage-associated genes including *TNF*, *CXCL11*, *PTGS2/COX2* (associated with inflammation), *IL1Ra*, *IDO*, *IL10* (immunoregulation), *DC-SIGN*, *MRC1* (scavenging) and *FNI*, *PDGFD*, *MMP12* and *TIMP1* (remodeling). We also stimulated models without monocytes implanted to determine the monocyte-associated effect on the gene expression of interest. Colleagues of ours previously employed this multiplex assay for the analysis of the cells in bronchoalveolar lavages, where the different genes identified distinct activation profiles that were influenced by the airway microbiota (302). The oral mucosa models with monocytes that were stimulated with the combination of LPS and IFN γ had increased gene expression of inflammatory (*COX2* and *TNF*) and tissue destructive (*MMP12*) components, compared to the un-stimulated models with monocytes (Fig. 5A-C, **paper I**). Stimulation with LPS only, resulted in decreased expression of *MRC1* and *IL10* (Suppl. figure 3, **paper I**). The models without monocytes had nearly undetectable expression of myeloid cell markers such as *DC-SIGN/CD209*, *MRC1/CD206* and *IL10*, and interestingly the gene expression in models without monocytes

did not change much after stimulation (Fig. 5A-D and suppl. figure 3, **paper I**). Induced tissue inflammation in models with monocytes was confirmed by the quantitation of MMP12 and TNF at a protein level in culture supernatants using ELISA, and in extracted cells by the detection of intracellular COX2 using flow cytometry (Fig. 5E-H, **paper I**). We concluded that the genes analyzed in the multiplex assay can provide information about distinct activation profiles in tissue, and therefore it was also employed to reveal differences in gingival tissue comparing individuals with or without PD.

To confirm that monocyte-derived cells are essential for the inflammatory-induced MMP12 production in oral mucosa models (Fig. 5A and E, **paper I**), we digested the tissue models and performed cytopins and IF staining for MMP12 in combination with CD68 or vimentin. This analysis demonstrated that MMP12 expression was associated with CD68⁺ monocyte-derived cells rather than vimentin⁺ fibroblasts or epithelial cells, which are negative for both CD68 and vimentin (Figure 14). Thus, these findings are also in line with our *ex vivo* analysis of PD tissue identifying CD68⁺CD64⁺ cells as the predominant source of MMP12 in PD gingival tissue (Fig. 3B, **paper I**).

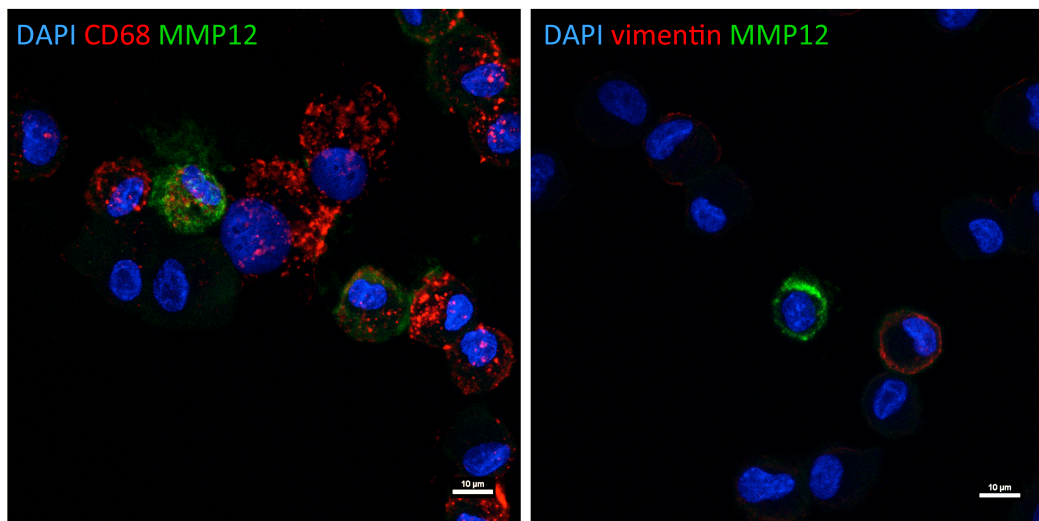


Figure 14. Immunofluorescence images on cells from digested oral mucosa models, stained for MMP12 (green) and to the right CD68 (red) and to the left vimentin (red).

Using another multicellular model system researchers found that LPS stimulation resulted in increased production of MMP3 and MMP9 by co-cultures of macrophages and gingival fibroblasts (303). Although not previously proven, this may indicate that tissue-derived components predispose and/or induce MMP production by monocyte-derived cells and vice versa. For example, in a skin model, activated monocyte-derived DCs induced the production of MMPs by the fibroblasts, which facilitated the migration of the DCs (86). Another example of tissue-mediated effect on myeloid cells is the study showing the induction of CCL18 in DC residing in the 3D lung tissue model, compared to DCs or models alone (136). These are examples on how important the crosstalk between myeloid immune cells and tissue constituent cells might be to orchestrate tissue inflammation. To assess the effect of tissue inflammation on the production of CSF1, IL-34, and CSF2, which are important growth and

differentiation factors for monocytes, we processed tissue models for CSF1, IL34, and CSF2 mRNA analyses. Of these three, it was above all CSF2 mRNA expression that was induced in oral mucosa models stimulated with LPS and IFN γ (Figure 15). Later on, we found that LPS on its own was sufficient to induce increased CSF2 mRNA expression in tissue models (Fig. 5C, **paper I**). CSF2 is indeed considered to be an inducible growth factor associated with tissue inflammation and survival of myeloid cells in barrier tissues, while CSF1 and IL-34 belong to a group of growth factors constitutively produced at different tissue sites, and that are important for the longevity of embryonically-derived macrophages (58, 102, 148, 304). However, when CSF1 is induced at sites where it is not detected during steady state, such as epidermis, it can contribute to tissue inflammation (305). CSF2 has been reported, at least *in vitro*, to induce an inflammatory macrophage phenotype (118, 304). Thus, stimulation of tissue models with TLR ligands induces tissue inflammation, with the potential to affect the phenotype and function of monocytes and monocyte-derived cells (**paper I and III**).

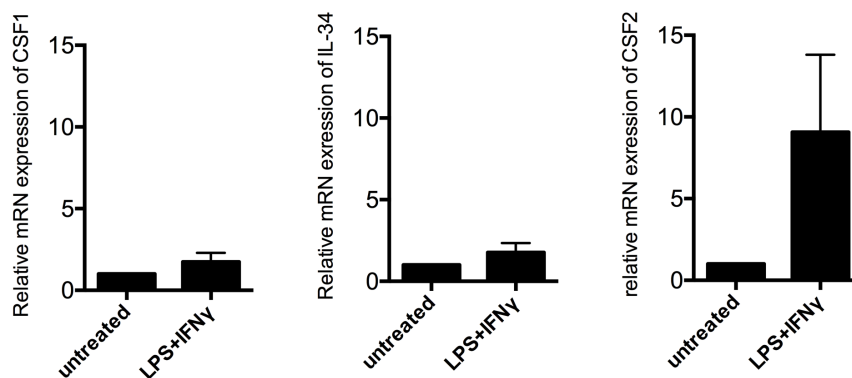


Figure 15. Real-time qRT-PCR analysis of the mRNA expression of the growth factors CSF1, IL-34 and CSF2, produced by the oral mucosa models with monocytes from three different donors in response to a 24 h stimulation with LPS and IFN γ . Bars represent mean \pm SD.

Considering the importance of the microbiota in initiating the inflammatory reaction in gingiva, it is also important to study the cross talk between the microbiota and host cells. This, Bao and colleagues recently showed by stimulating tissue models with an 11 bacterial-species biofilm. The tissue responded with production of inflammatory cytokines and was able to reduce the number of several of the bacterial species (292). Another alternative is to culture the models with saliva or dental plaque from healthy and PD individuals to study the influence of different microbiota and host-derived components on tissue inflammation (210). Yet we have not performed these types of studies, but our newly developed tissue model systems can enable such studies. Although there are many benefits of the developed 3D tissue models, of course, there are also shortcomings. This includes among other things the use of cell lines, which over time could be replaced by primary cells from healthy and diseased tissues, which better mimic a normal tissue environment. We have not yet developed a system for mixing different types of immune cells, such as monocyte-derived cells and T cells, which would allow us to study the interaction between these cells in a tissue like setting. It is also critical to stress that these model systems are a simplification of normal tissue, which have additional components, both cellular and non-cellular, such as

vasculature. Finally, it is important to recall that these models can be complementary to patient sample analyzes and experimental *in vivo* models.

4.2 INFLAMMATORY SIGNATURE IN PD GINGIVA

Secondly, we aimed to study inflammatory signatures associated with macrophage functions in PD gingival tissue. In order to do this, we applied the same multiplex assay as for the oral mucosa models, and the expressions of the 12 genes associated with distinct macrophage tissue functions were compared between PD and control patients. This approach was used to identify the activation profile as well as a screening tool to identify targets for further analysis and potential immunomodulatory therapies.

4.2.1 Gene expression in PD gingiva reflect increased inflammatory and tissue destructive activity

Gingival tissue from 18 patients with PD as well as from 14 controls without PD (**table 1, study I**) were analyzed with multiplex real-time PCR for the expression of *TNF*, *CXCL11*, *COX2* (associated with inflammation), *IL1Ra*, *IDO*, *IL10* (immunoregulation), *DC-SIGN*, *MRC1* (scavenging), and *FNI*, *PDGFD*, *MMP12* and *TIMP1* (remodeling). The genes up-regulated in gingiva from PD patients were *MMP12*, *COX2*, *TNF* and *DC-SIGN*, while *TIMP1* and the other analyzed genes were unaltered (Fig. 5A and suppl. figure 3, **paper I**). Lately transcriptomics have been performed on gingival tissue from diseased and healthy sites in patients with PD (306-308). The approach to compare diseased and healthy sites in the same individual I think can be a good way to identify genes associated with inflammatory reactions in the gingiva. However, the individual gene levels might be high to start with also in “healthy” gingiva from individuals with PD, with the risk of masking genes associated to PD. In our study, we focused on a selection of genes and compared their expression in gingival tissue of patients with PD and controls without PD. We think that the comparison of gene signatures in individuals that are susceptible to PD versus individuals that do not develop pathological disease is advantageous to identify PD associated alterations. However, a drawback could be inter-individual variations, where the gene expression levels in one individual with disease not necessarily leads to pathology in another individual.

Similar to our approach of comparing gingiva from PD and control individuals, RNA sequencing have been performed recently by another group, and identified functions related to inflammation and injury as the most highly up-regulated functions in PD gingiva (248). That study also identified another MMP, the MMP7 to be associated with PD. Additional MMPs have been associated with the tissue destruction in PD, and MMP8 and MMP9 are suggested to be potential biomarkers for PD (309, 310). Though little is known about the macrophage metalloelastase, MMP12, in PD affected gingival tissue. A study on juvenile localized aggressive PD analyzed the effect of periodontal treatment on the level of MMP12 in GCF, and identified a decrease in levels accompanied by improved periodontal parameters (311). Uncontrolled MMP12 production can lead to pathological tissue degradation and the increased release of membrane-bound TNF (312). In line with this, gingival tissue biopsies

from PD patients had significantly lower tropoelastin, one of the substrates for MMP12 (Fig. 8, **paper I**). MMP12 can also attack other tissue structural proteins, as was visualized in the oral mucosa model treated with exogenous MMP12 and where the level of fibronectin was reduced (87, 88, 313) (Fig. 7, **paper I**). In another study, the expression of MMP12 was shown to be induced by orthodontic treatment, and was associated with degradation of the basement membrane component collagen-IV in the tension zone in the periodontal ligament tissue (314). In addition to the direct effects on the ECM proteins, MMP12 can also activate other MMPs, such as MMP3 and MMP9, both implicated in the pathogenesis of PD (307, 309, 315). An MMP12 knock out mouse model also showed reduced tissue recruitment of macrophages (315). The effect of MMP12 on cell migration could be due to its ability to degrade ECM proteins or via the ability of MMP12 to induce chemokine production by other cells, such as epithelial cells (316). MMP12 can also be expressed by osteoclasts and is able to degrade bone matrix proteins, which in turn activates the osteoclasts (317). Several MMPs have been associated with PD and may explain the tissue destruction observed, and our finding of increased MMP12 in PD gingival tissue add to the list of MMPs linked to PD (183, 318-321) (**paper I**). In addition, the conditions that we and others have observed in the gingival tissue of PD, likely results in increased production of inflammatory cytokines that leads to aggravated inflammation and a subsequent loss of tooth supportive structures (322).

4.2.2 Monocyte-derived cells in PD gingiva display increased tissue destructive activity

In order to delineate the cause of increased MMP12 production in PD gingival tissue, we next sought to identify the cellular source. Gingival tissue from individuals with PD and controls were enzymatically digested and analyzed by flow cytometry. The flow cytometry analysis identified a CD68⁺CD14⁺CD64⁺ cell population to be responsible for the increased MMP12 production in PD (Fig. 3B, **paper I**). Based on the expression of CD68, CD64 and CD14, we considered these cells to be monocyte-derived cells (52, 160, 323, 324). Another interesting finding we did when analyzing the CD68⁺CD14⁺CD64⁺ cells in PD was the reduced surface expression of the co-inhibitory molecule, CD200R (Fig. 3C, **paper I**). The down-regulation of CD200R was in line with the phenotype of the monocyte-derived cells in the inflamed oral mucosa models (Fig. 4E, **paper I**). Other studies on chronic inflammatory diseases have also identified reduced CD200R expression by monocyte-derived cells and macrophages (120, 121, 123, 325, 326). The reduced CD200R expression has been associated with an inflammatory phenotype and chronic inflammation (120, 121, 123, 325, 326). Macrophage-like cells have also been linked to gut inflammation, and suggested to originate from classical monocytes based on their CD14^{high}CD16^{low} expression and their ability to produce MMPs (323). Monocyte-derived cells produce several MMPs already at steady state conditions, and inflammatory as well as microbial stimuli further induce their MMP producing capacities (107, 108). The steady state production of MMPs by monocyte-derived macrophages could be a mechanism for homeostatic tissue remodeling. However, increased expression and production of MMPs is associated with immunopathology in chronic inflammatory diseases, and may be potential targets for intervention (200, 327).

4.3 UNDERSTANDING AND MODULATING MONOCYTE MMP12-PRODUCING CAPACITIES

Next, we utilized our developed oral mucosa models with monocyte-derived cells as well as monocultures with monocyte-derived cells, to delineate inflammatory pathways and especially MMP12 regulation.

4.3.1 COX2-mediated tissue inflammation and MMP production

The 3D tissue models can be utilized to explore pathways and mechanisms to modulate the increased MMP production in chronic tissue inflammation. Tissue inflammation is associated with inducible COX2, which is expressed in response to injury, as well as inflammatory and microbial mediators (328). COX2 is an enzyme involved in the conversion of arachidonic acid into prostacyclins, thromboxanes and the prostaglandins (PG), such as PGE₂, which exert different inflammatory and homeostatic functions (329). The induction of COX2 and subsequent production of PGE₂ have been shown to induce the production of several MMPs, such as MMP2 and MMP9, though it was not known if it modifies the production of MMP12 (330, 331). In PD, increased levels of PGE₂ have been observed and a role in bone degradation is suggested (332, 333). To test if the COX2/PGE₂ pathway controls MMP12 production by monocyte-derived cells in a tissue setting, we utilized our established oral mucosa model in combination with a small-molecule COX2 inhibitor. The models were stimulated with LPS, which was shown to induce MMP12 production in the oral mucosa models (Fig. 5E and 6D, **paper I**). In a set of stimulated models, we also added the COX2 specific inhibitor, SC-58125, and incubated the models for 24 h (Figure 16). The culture supernatants from stimulated oral mucosa models were subsequently analyzed for the production of PGE₂ and MMP12. The production of PGE₂ was robustly inhibited, demonstrating the efficiency of the COX2 inhibitor (Fig. 6A, **paper I**). In contrast to PGE₂ production, the production of MMP12 was unaffected (Fig. 6B, **paper I**). Therefore we concluded that the COX2/PGE₂ pathway is not involved in the production of MMP12 by the oral mucosa model. Besides giving us this valuable information, this experiment also highlight the usefulness of the 3D mucosa models in testing targeted interventions aimed at modulating tissue inflammation.

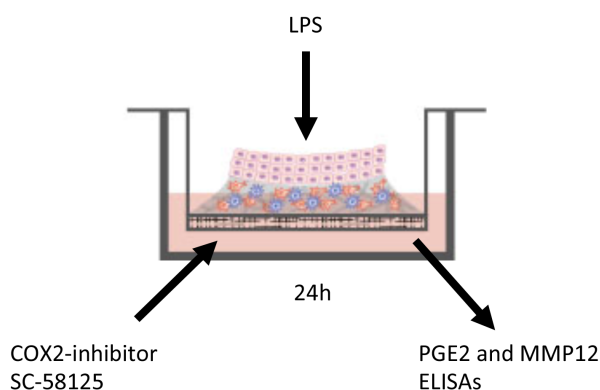


Figure 16. Illustration of the approach we used for the COX2-inhibition experiment. Oral mucosa models with monocytes implanted were stimulated with LPS the last 24 h of the culturing period, in combination with a COX2-inhibitor treatment in the culture medium. The PGE₂- and MMP12-production by the oral mucosa models were analyzed with ELISAs. *Illustrated by Puran Chen.*

4.3.2 Tissue inflammation and CSF2-induced MMP12

Although, low levels of CSF2 can promote the survival of monocyte-derived cells and tissue-resident macrophages in certain tissues, higher levels of CSF2 is associated with tissue inflammation and induces inflammatory functions in these cells (151, 252, 304). Increased levels of CSF2 have been associated to chronic PD and we also observed increased *CSF2* expression in the stimulated oral mucosa models (Figure 15; Fig. 6C, **paper I**) (334). Human primary monocytes were cultured with CSF1, which is constitutively produced in most tissues including the oral mucosa model, to generate monocyte-derived macrophage-like cells. Subsequently, the monocyte-derived cells were treated with CSF2 for 24 h and the MMP12 production was assessed by ELISA. This revealed a strong induction of MMP12 production by monocyte-derived cells (Fig. 6D, **paper I**). In line with our findings, CSF2 induced the production of MMP12 by primary monocytes and the U937 monocytic cell line, and IL-1 β and CCL2 further enhanced the production (335). Treatment of mice with a CSF2 blocking antibody reduced the levels of MMP3, MMP9, MMP13 and MMP14, which were adversely induced by an injected CSF2 vector (336). In a 3D tumor model, enhanced CSF2 expression resulted in increased expression of MMP2, MMP9, and MMP26, and an altered basement membrane, which was restored by CSF2 blocking antibodies (337). These findings suggest that CSF2 has effects beyond MMP12 by inducing several other MMPs as well.

4.3.3 The CD200/CD200R pathway modulates MMP12 production in monocyte-derived cells

The CD200 and CD200R signaling is important for the control of myeloid cells in different tissues (338). It has been shown that exogenous CD200 treatment up-regulates the expression of CD200R and down-regulate TLR4, rendering the cells less responsive to LPS stimulation and inhibiting the production of pro-inflammatory cytokines (339). The reduced CD200R expression on the monocyte-derived cells in PD and the inflammatory oral mucosa models (Fig. 3C and 4F, **paper I**), lead us to speculate that the CD200/CD200R pathway could also affect MMP12 production in monocyte-derived cells. We speculated that treatment with CD200 could potentially attenuate the CSF2-induced MMP12 production, and indeed, treatment of the monocyte-derived cell with a recombinant human CD200 Fc chimera protein lead to reduced CSF2-induced MMP12 production (Fig. 6E, **paper I**). The exact molecular mechanisms behind CD200/CD200R-mediated attenuation of MMP12 production in monocyte-derived cells are not known, and therefore it is only possible to speculate. The CD200/CD200R ligation induces phosphorylation of downstream of tyrosine kinase 2 (Dok2) and the recruitment of Ras GTPase activating protein (RasGAP) that in turn inactivates the Ras signaling pathway (340, 341). The Ras signaling pathway is upstream of both phosphatidylinositol 3-kinases (PI3K) and Raf, which are upstream of the MAPKs that in turn controls the activator protein 1 (AP-1) motif, consisting of Jun-Fos combinations (341). AP-1 has been shown to regulate the transcription of MMP12 in smooth muscle cells and in U937 monocytes (335, 342). In addition, CSF2 has been shown to activate the Ras signaling pathway via JAK-STAT activation (343). Therefore, we think that the CSF2 and CD200R

can affect the Ras signaling pathway in different ways leading to increased or decreased AP-1 regulated MMP12 production (Figure 17). The classical monocyte subset which is the main monocyte subset speculated to be recruited to inflamed tissues, have the highest AP-1 gene expression (49). In addition, the JAK-STAT, PI3K and Ras signaling pathways are shown to be up-regulated in diseased gingival tissue from individuals with PD (307, 344). However, the expressions of CD200R have not been addressed in PD prior to this study, and just as little is known on AP-1. Nevertheless, a decreased CD200R expression has been associated to RA, which is another chronic inflammatory diseases that involves degradation of bone, and in line with this, the CD200/CD200R pathway can inhibit osteoclastogenesis (122, 326).

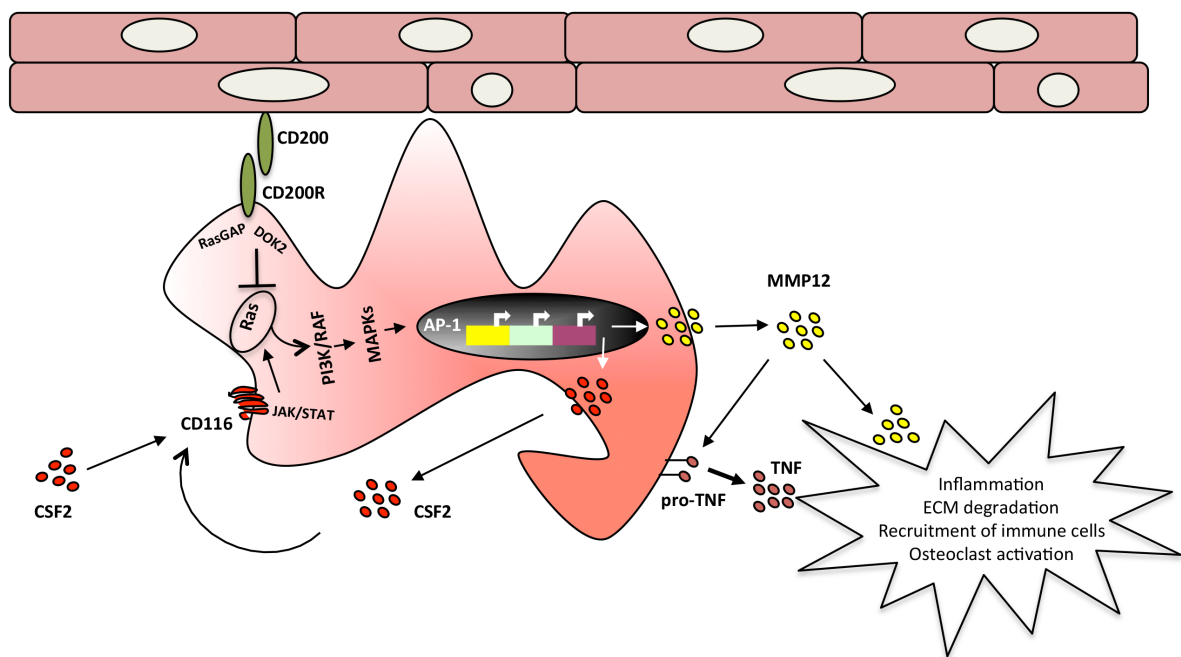


Figure 17. Illustration of the results in **Study I**, and speculations on mechanisms in relation to: (122, 312, 339, 340, 342, 343). RasGAP, Ras GTPase activating protein; Dok2, downstream of tyrosine kinase 2; PI3K, phosphatidylinositol 3-kinases; MAPKs, mitogen-activated protein kinases; AP-1, activator protein 1. Illustration by Sofia Björnföt Holmström and Mattias Svensson.

4.4 MONOCYTE-ASSOCIATED SALIVARY BIOMARKERS

Diagnostics of PD is today based on clinical parameters, which are useful to identify the active progressed disease. However, the clinical parameters are insufficient for early diagnosis, and to provide information about susceptibility and prediction of disease progression. Identifying altered immune responses with increased risk of severe immunopathology might help in directing the treatment of PD and other chronic inflammatory diseases. Saliva has recently been introduced as a diagnostic tool in dental care and has several benefits compared to GCF, gingival biopsies and blood samples, since it is non-invasive, easy and fast to collect (345). Saliva also allow for the collection of a relatively large sample volume and the detection of proteins that are expressed in plasma and associated to systemic diseases (346). However, there are some draw backs with saliva as relatively high

viscosity and it is less specific for periodontal diseases than GCF, and gingival tissue (346). Overall, however, there are more advantages than disadvantages using saliva samples and we chose to try to analyze the salivary levels of MMP12, the S100A8 (calgranulin A) and S100A9 (calgranulin B) complex, referred to as calprotectin or as MRP-8/14, and S100A12 (calgranulin C), all associated to monocytes and monocyte-derived cells (39, 108, 335, 347-349).

4.4.1 Salivary MMP12, S100A8/A9, and S100A12 in PD

In total, 436 individuals were clinically examined, asked to fill in a health questionnaire, and saliva samples were collected and subsequently analyzed for the levels of MMP12, S100A8/A9, and S100A12. The demographics of the individuals are presented in **Table 1** in **paper II**, and the clinical examinations and health questionnaire are described in detail in Lundgren et al 2012 (288). This study allowed for determination of the influence of oral and systemic diseases, as well as of non-disease related covariates, such as age, sex and smoking, on the levels of MMP12, S100A8/A9, and S100A12. To identify the explanatory variables for the biomarker candidates, we applied a multivariate regression analysis. This analysis identified that the percentage of sites with a PPD ≥ 4 mm, age, smoking and the presence of tumor (self-reported) to be explanatory for the MMP12 levels, and could explain 27.6 % of the variance (Table 2, **paper II**). A decrease in MMP12 levels in saliva were found in smokers and with age, while increased salivary MMP12 levels were associated with percentage of sites with a PPD ≥ 4 mm and the presence of tumor (self-reported) (Table 2, **paper II**). The decrease in MMP in smokers can possibly be explained by an altered monocyte and macrophage phenotype leading to decreased MMP12 production in response to chronic smoke exposure (350). In addition, smoking also results in decreased GCF production, which also could explain the reduced MMP12 levels in saliva in smokers (351).

The only explanatory variable for S100A8/A9 was the percentage of sites with bleeding on probing (BOP), which accounted for 22.9 % of the variance. The levels of S100A12 were associated with percentage of sites with BOP and the presence of tumor (self-reported), which together accounted for 22.6 % of variance (Table 2, **paper II**). We also found that the levels of MMP12, S100A8/A9 and S100A12 correlated strongly with each other (Fig. 3, **paper II**). To the best of our knowledge, this study was the first to evaluate MMP12 and S100A12 as potential salivary biomarker candidates for PD. Nonetheless, increased levels of MMP12 and S100A12 in GCF have recently been associated to localized aggressive PD and chronic PD, respectively (311, 352). Calprotectin (S100A8/A9) was suggested to be a potential biomarker for PD in a recent review and has been associated to PD in former studies on salivary levels, however the sample sizes were rather small (353-355). In addition, periodontal treatment was shown to reduce the levels of calprotectin in saliva (356).

The S100s belong to the DAMPs and can bind to TLR4 and receptor for advanced glycation end products (RAGE) to activate proinflammatory signaling cascades (349). The S100A8 and S100A9 can be produced by CSF2-differentiated macrophages and have been shown to

induce the production of MMPs and proinflammatory cytokines (347). In addition, S100A8 and S100A9 can induce migration of tumor cells, probably via the increased production of MMP2 and MMP12 (357). Calprotectin also induces the production of pro-inflammatory cytokines by macrophages in RA (348), and calprotectin and S100A12 have been suggested as biomarkers for RA (358). Increased levels of S100A8/A9 has also been linked to osteoarthritis (OA) in a mouse model, and resulted in increased recruitment of the Ly6C^{high} monocytes resembling the classical monocytes in human (359). The high levels of S100A8/A9 in OA could be due to increased production by infiltrating classical monocytes and monocyte-derived cells. This is supported by the fact that human classical monocytes express high levels of S100A8, S100A9, and S100A12 mRNA (39, 49).

Besides the markers analyzed by us, there are also several other markers associated with innate immune responses that have shown promising results as candidate biomarkers for PD, such as CCL2, CCL3, IL-1 β , IL-6, MMP8, MMP9, CSF1, RANKL and STREM-1 (310, 320, 353, 360-363). Considering that PD is a multifactorial complex disease, a combination of host-derived and microbial markers is suggested to provide accurate diagnosis, treatment and risk assessment (364-366). To the panel of markers mentioned, we would like to suggest MMP12, S100A8/A9 and S100A12, which reflect different aspects of periodontal inflammation, for further investigation.

4.5 IL-17A EXPRESSION IN MONOCYTES

In several chronic inflammatory diseases affecting different types of tissues, increased gene expression and protein levels of the cytokine IL-17A have been identified, and linked to the immunopathology (163, 170, 227, 261, 367-369). In line with this, therapeutic agents targeting IL-17A have shown potential in several chronic inflammatory diseases (218). IL-17A can initiate inflammatory reactions, granuloma formation and osteoclast activation, either directly or via inducing chemokines, cytokines, and the MMPs (162, 163, 224, 246, 261). Besides contribution to chronic tissue inflammation, IL-17A is important in the control of the commensal microbiota and the maintenance of barrier functions in epithelial tissues (206, 370). Cells of lymphoid origin, such as T_H17 cells, $\gamma\delta$ T cells, ILC3s, NK T cells, and lymphoid tissue inducer (LTi) cells, are mainly described as producers of IL-17A at steady state as well as under inflammatory conditions (170, 371). However, it has also been shown that cells of myeloid origin can produce IL-17A in inflamed tissues, such as the monocyte-derived DC in LCH lesions (163, 260, 371). Though it is not known if the blood monocytes, which are precursors for monocyte-derived DC and potentially also for the LCH specific multinucleated cells, produce IL-17A already in circulation. Therefore, **Study IV** was conducted.

4.5.1 LCH monocytes produce IL-17A

To study the IL-17A expression in blood monocytes, we isolated PBMCs from healthy controls and patients with LCH, as well as from patients with PD, and performed flow cytometry and RT-qPCR analyses. The flow cytometry analysis revealed that almost the whole fraction of LCH monocytes was producers of IL-17A, while lymphoid cells were negative for IL-17A. Monocytes from healthy controls or patients with PD were also negative for IL-17A (Figure 18; Fig. 1A and 2A-B, **paper IV**). In line with our findings, plasma or serum from LCH patients contain increased levels of IL-17A (163, 261, 372). Increased levels of IL-17A have also been observed in PD plasma (162, 373). The increased levels of IL-17A in plasma in PD could potentially reside from leakage of inflammatory mediators in inflamed tissues into the blood stream, or from the lymphoid compartment in blood, however, the number of CD4⁺IL-17A⁺ T cells was not increase in PD blood compared to controls (244). At the time of our study there was a controversy in the field whether the reagents used for the detection of IL-17A protein with ELISAs and WB specifically recognized IL-17A and therefore such analyzes were not performed. Later, however, plasma analyzes have been performed on this cohort, analyzing both cerebral spinal fluid and plasma, and there are indications that elevated levels of IL-17A accompany disease activity (unpublished observation and (264)).

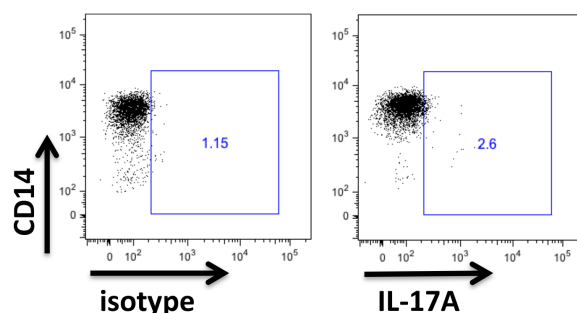


Figure 18. Flow cytometry analysis of the intracellular IL-17A expression in the monocytes from a patient with PD.

In addition, the analysis of IL-17A mRNA expression in PBMCs and monocytes from controls, LCH and PD patients revealed that the LCH monocytes had the highest IL-17A mRNA expression (Fig. 3A, **paper IV**). Since we were also interested in knowing which types of cells could respond to IL-17A in the circulation, we analyzed the expression of IL-17RA on leukocytes in blood. This identified that the majority of monocytes as well as subpopulations of lymphocytes express the IL-17RA on the cell surface (Fig. 1, **paper IV**). In a study comparing single and multiple system LCH, increased levels of the IL-17RA was identified in lesions in patients with multiple system LCH (261).

4.5.2 Mechanisms of IL-17A production by LCH monocytes

TLR stimulation induces the production of IL-23 by monocyte-derived cells, neutrophils, and epithelial cells, which in turn can stimulate the production of IL-17A by cells expressing the IL-23R (217, 219, 244, 374). In line with this, we showed that stimulation with IL-23 or with phorbol myristate acetate and Ionomycin induced elevated IL-17A mRNA expression in monocytes (Fig. 3E, **paper IV**). The synthesis of IL-17A in lymphoid cells is regulated by

the transcription factor ROR γ t (encoded by *RORC*) (371). To verify the existence of this pathway also in blood monocytes producing IL-17A, the IL-17A-positive monocytes were analyzed for their expression of ROR γ t by flow cytometry. Indeed, the LCH IL-17A⁺ monocytes expressed ROR γ t (Fig. 2C, **paper IV**), and in line with this the LCH monocyte mRNA expression for *RORC* was significantly increased compared to controls and the monocytes from PD patients (Fig. 3B, **paper IV**).

The pathological IL-17A production by LCH monocytes can potentially be explained by recently discovered mutations in the *BRAF* gene in LCH lesions that is involved in the Ras-RAF-MEK-ERK signaling pathway, which regulates among others the production of IL-17A (256, 257, 375). The mutation has been identified in blood monocytes and cDC in patients with LCH, and suggests that LCH has an neoplastic component causing the inflammatory disorder (255, 257). Targeting the *BRAF* or the whole fraction of monocytes in LCH, have shown promising results *in vitro* and *in vivo* (264, 376). To study the effect of a specific *BRAF* mutation, V600, on monocyte function including IL-17A production, we are currently working on optimizing a protocol for transducing human primary monocytes with *BRAF*^{V600}. In addition to analyzing the direct effect of introducing *BRAF*^{V600} into monocytes, we will also implant such monocytes in our 3D tissue models for the purpose of studying the impact on tissue inflammation, remodeling and pathology.

5 CONCLUDING REMARKS AND FUTURE ASPECTS

Chronic inflammatory diseases affect a large number of individuals worldwide and the immunopathology that results from the inflammatory reactions cause irreversible tissue damage, leading to decreased functionality and quality of life for the individuals affected. By increasing the understanding on the role of monocytes in chronic inflammatory diseases, we anticipated to identify mechanisms by which these cells can be modulated or targeted, in order to attenuate the inflammation and subsequent immunopathology.

The main findings and achievements in my thesis work were:

- PD gingiva presented with an increased inflammatory (COX2, TNF and DC-SIGN) and tissue remodelling (MMP12) activity
- Monocyte-derived cells from PD gingiva were identified as the main producers of MMP12, accompanied by decreased CD200R surface expression
- The CD200/CD200R pathway was shown to be involved in the regulation of MMP12 production by monocyte-derived cells
- Inflamed 3D tissue models of oral and lung mucosa were developed to study human monocyte-derived cells in a physiologically relevant milieu
- The levels of MMP12 in saliva were associated with increased percentage of gingival pockets with pathological depths
- S100A8/A9 and S100A12 levels in saliva were associated with increased gingival inflammation
- Blood monocytes from LCH patients produced IL-17A

These findings and accomplishments have identified functions and pathways, as well as established methods that form the basis for future studies aimed at developing immunomodulatory treatments of chronic inflammatory diseases. However, there is still much to learn about the various monocyte subsets found in humans, and we believe that our newly developed tissue model systems can be valuable tools in such studies. As mentioned earlier, it would be interesting to sort and implant the three distinct monocyte subsets into the tissue models, and study their respective survival capacity and functional behavior in response to various stimuli. We believe that the 3D tissue models can be used to study how specific mutations in monocytes impact on tissue inflammation and homeostasis. In addition, 3D models with a combination of monocytes and other immune cells, such as tissue-resident macrophages, could give information about the interplay and the distinct roles the respective cells have during responses to microbial stimuli and the resolution phase of inflammation. In this context, it would also be interesting to introduce, for example, fibroblasts from individuals with specific diseases and study the effect on the innate immune cells.

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